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PROTEOLYTIC ENZYMES AND THEIR CLINICAL APPLICATION

BY

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PROTEOLYTIC ENZYMES AND THEIR CLINICAL APPLICATION*

Consulting Editor and Conference Chairman
GUSTAV J. MARTIN

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* This series of papers is the result of a conference on *Proteolytic Enzymes and Their Clinical Application* held by the Section of Biology of The New York Academy of Sciences, November 8 and 9, 1956.

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Part I. Laboratory Studies

ISOLATION, PURIFICATION, AND PROPERTIES OF PROTEOLYTIC ENZYMES FROM ANIMAL SOURCES*

By Felix Haurowitz

Department of Chemistry, Indiana University, Bloomington, Ind.

Since proteolytic enzymes and their actions will be discussed at length in this monograph, it might be desirable at the outset to define these enzymes clearly and to classify them logically. In both respects we encounter great difficulties. Originally, the true proteases, which catalyze the hydrolysis of proteins, were differentiated from the peptidases, which catalyze the hydrolysis of polypeptides. It was not clear at that time whether the proteases were able to catalyze the hydrolysis of peptide bonds. This reaction was demonstrated first by Bergmann and his co-workers,[1] who succeeded in preparing peptides that were split by the action of pepsin, trypsin, and other typical proteases. Accordingly, the differentiation between proteases and peptidases was abandoned, and a new classification as exopeptidases and endopeptidases was proposed. The exopeptidases were defined as enzymes that catalyze the hydrolysis of only those peptide bonds that are formed by the N- or C-terminal amino acids, whereas endopeptidases were considered as enzymes that catalyze the hydrolysis of peptide bonds inside the peptide chain. Although this classification is frequently used at present, it is not absolutely valid. Thus pepsin, a typical endopeptidase, is able to catalyze the hydrolysis of cysteyl-tyrosine. Difficulties in the nomenclature and classification of the proteolytic enzymes arose also when it was discovered that chymotrypsin catalyzes, not only the hydrolysis of peptide bonds, but also that of ester bonds[2] and carbon-carbon bonds.[3] Evidently a systematic classification of the proteolytic enzymes cannot be based on the nature of the hydrolyzed bonds.

It would be desirable to correlate the classification of the proteolytic enzymes with the specific structure of their active groups. Unfortunately, we know very little about the nature of these groups. Certain differences between these groups are revealed by the fact that different proteolytic enzymes require different types of activators. Accordingly, E. L. Smith[4] has proposed to classify the proteolytic enzymes as three groups: (1) enzymes that do not require any activator; (2) those that need activation by cyanides or sulfides; and (3) those that require activation by metal ions. I shall not dwell further on the problem of classification, since it does not help us to attain more insight into the nature of the proteolytic enzymes.

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* The experimental work on plastein formation carried out in the author's laboratory and described in this article was supported by grants from the National Science Foundation, Washington, D. C., and the United States Public Health Service, Bethesda, Md., and by contracts of Indiana University, Bloomington, Ind., with the United States Office of Naval Research and the United States Atomic Energy Commission, Washington, D. C.

The most important of the proteolytic enzymes of animal origin are pepsin, trypsin, chymotrypsin, cathepsin, and various exopeptidases from the intestinal juice. In spite of great differences between the chemical and physical properties of these enzymes, the methods used for their isolation and preparation are based on similar principles. In most instances fractionation by salting-out with ammonium sulfate has been used. This classical technique has been improved to perfection in the hands of Northrop *et al.*,[5] who modified it by varying *p*H, temperature, and other factors. Using this method Northrop *et al.*[5] succeeded in isolating many of the proteolytic enzymes in the crystalline state. High concentrations of ammonium sulfate are not only useful as fractionating agents, but they seem also to protect the enzymes against denaturation.

It is remarkable that many of the proteolytic enzymes are rather stable at elevated temperatures. It is possible, therefore, to denature contaminating proteins by short exposure to temperatures of approximately 90° C. and to remove the denatured, insoluble contaminants by filtration or centrifugation. The heat stability of these enzymes, as well as their great tendency to crystallize, is probably correlated with their low molecular weights, which are in the range between 20,000 and 40,000. Their great heat resistance seems also to indicate that the conformation of the peptide chains is rather stable and rigid and that the tendency to rearrangements is low.

Pepsin, trypsin, chymotrypsin, and carboxypeptidase are present *in vivo* as inactive proenzymes or zymogens. Activation is accomplished by limited proteolysis of the proenzyme molecules. This proteolysis can be brought about by traces of the active enzyme or by other enzymes. It seems that activation in all instances is accompanied by the cleavage of few peptide bonds. In the activation of trypsinogen a hexapeptide is split off.[6] In the activation of chymotrypsinogen we are dealing with the stepwise elimination of a dipeptide whereas, in the activation of pepsinogen, a large peptide molecule of a molecular weight close to 5000 is removed. I have mentioned the activation mechanism because it plays an important role in the isolation of the proteolytic enzymes.

Before discussing the isolation and purification of the proteolytic enzymes, it may be useful to discuss briefly the methods employed for the quantitative measurement of enzyme action. Originally, proteolysis was measured by determining the increase in amino or carboxyl groups. This can be accomplished by the Van Slyke method of amino-group determination, by Sörensen's formol titration, or by electrometric titration. These methods are very satisfactory when we are dealing with large increases in amino or carboxyl groups; they are less suitable for microanalyses and have been replaced by the colorimetric determination of phenol groups in the protein or nonprotein fractions or by spectrophotometry in the ultraviolet range. Both optical methods are based on the high absorbancy of the aromatic amino acids and they fail, therefore, when the substrate is devoid of such amino acids. One of the most sensitive techniques for measuring proteolytic breakdown is the ninhydrin method, which has been promoted to a quantitative method by the work of Stein and Moore.[7] Another reagent of great value is 2,4-

dinitrofluorobenzene, which converts amino acids and peptides into yellow dinitrophenyl[8] derivatives and thus enables us to determine them by optical methods. In many instances changes in the viscosity, solubility, or other physical properties of proteins have been used as criteria for the quantitative determination. It is not always possible to correlate them with a definite change in the number of hydrolyzed peptide bonds.

Linderstrøm-Lang[9] found that the action of proteolytic enzymes frequently causes an initial contraction of the volume of the protein solution. It has not yet been possible to decide whether this is caused only by the hydrolytic cleavage of peptide bonds or also by other changes in the structure of the protein substrate. We are confronted with a similar phenomenon when we investigate the action of clotting enzymes. Although we know that some, if not all, of these are proteolytic enzymes, it is not yet clear how many peptide bonds are split during the clotting process and whether the hydrolytic breakdown of peptide bonds in this reaction is not accompanied by the formation of new peptide bonds—in other words, by transpeptidation. We speak customarily of proteolytic enzymes, but each of these enzymes is also able to catalyze the *formation* of new peptide bonds. This possibility complicates the interpretation of the volume changes accompanying the initial phases of proteolysis and obscures the mechanism of the clotting reaction to such an extent that it is hardly possible to use determinations of amino or carboxyl groups as a measure of the clotting action of proteolytic enzymes.

Pepsin, the proteolytic enzyme of the gastric juice, is purified by precipitation with copper hydroxide; the precipitate is decomposed by phosphate and the soluble enzyme is separated from insoluble copper phosphate by centrifugation. Pepsin is a highly acidic protein, very deficient in basic amino acids. Its molecule contains only 2 residues of arginine, 2 of lysine, and 2 of histidine, but approximately 37 residues of free glutamic or aspartic acid in addition to asparagine and glutamine residues. Accordingly, the isoelectric point of pepsin is at an unusually low *p*H value of less than 1.0. The hydrolytic action of pepsin may be correlated with this unusually high acidity. We see similar catalytic actions in resins that contain sulfonic acid residues. Thus, the hydrolytic cleavage of glycine peptides is catalyzed by Dowex 50 resin at 54° C. much more than by dilute HCl.[10] The *p*H optimum of pepsin is close to its isoelectric point, between *p*H 1 and 2. Pepsin is very stable at this high acidity, but is rapidly and irreversibly denatured and inactivated at *p*H values above 6.0.

The proteolytic enzymes of the pancreas are trypsin, chymotrypsin, and carboxypeptidase. Although trypsin has been known for a much longer time than chymotrypsin and the peptidase, we shall discuss chymotrypsin first because it is obtained prior to trypsin in the procedure of Northrop *et al.*[5] In this method the ice-cold pancreas is extracted with cold *N*/4 sulfuric acid, which extracts both chymotrypsinogen and trypsinogen. The former is salted out by ammonium sulfate between 40 and 60 per cent saturation at *p*H 5. When inoculated with traces of crystalline trypsin, chymotrypsinogen is converted into α-chymotrypsin. On prolonged standing at *p*H 8, α-chymotrypsin is converted into γ-chymotrypsin; from the filtrate,

β-chymotrypsin has been prepared. When chymotrypsinogen is incubated with larger amounts of crystalline trypsin, an unstable π-chymotrypsin is produced that later is converted into stable δ-chymotrypsin. Most of these isomers have been obtained in crystalline form. They differ from one another in their activities, their molecular weights, and other properties. It may be mentioned that another enzyme, called chymotrypsinogen B, and the corresponding chymotrypsin B have been isolated from pancreas; the isoelectric point of chymotrypsin B is at *p*H 4.7 whereas that of α-chymotrypsin is close to *p*H 8.[11] It is worthy of note that chymotrypsinogen is activated by trypsin, but not by chymotrypsin. Chymotrypsin catalyzes the hydrolysis of peptide, amide, hydrazide, hydroxylamide, and ester bonds, and catalyzes also the clotting of milk. Chymotrypsin is irreversibly inactivated by diisopropylfluorophosphate; in this interesting reaction one molecule of the inhibitor is bound to the enzyme molecule, probably to a seryl residue.

Trypsinogen, in the procedure of Northrop *et al.*,[5] is prepared from the mother liquors of the chymotrypsinogen preparation; it is salted out by increasing the ammonium sulfate concentration at a *p*H of 3.0. Trypsinogen is activated and converted to trypsin by trypsin or by enterokinase, an enzyme found in the intestinal juice. The isoelectric point of trypsin is at about *p*H 11, but is shifted to more acidic values by calcium salts. Trypsin has a thromboplastic effect; it accelerates the clotting process. It has also a weak chymotrypsin activity. Acetylation of trypsin[12] or treatment with sodium hypochlorite[13] has been recommended for the medicinal use of trypsin. Since trypsin is inactivated by treatment with hydrogen peroxide and since the inactivation parallels the decrease in ultraviolet absorbancy, it has been concluded that the active group of trypsin contains tryptophan residues.[14]

Pepsin, trypsin, and chymotrypsin are the most thoroughly investigated animal proteolytic enzymes. Much less is known about the other proteases. The proteolytic enzyme of the blood plasma reveals its presence by its fibrinolytic action; it has been called plasmin, and it will be discussed in this publication by D. L. Kline. Carboxypeptidase has been prepared in crystalline form from the pancreas by Anson;[15] it contains one zinc atom per molecule. Like chymotrypsin and trypsin, carboxypeptidase catalyzes the hydrolysis, not only of peptides and amides, but also that of ester bonds. Several aminopeptidases have been isolated by Smith and his co-workers[16] from the intestinal mucosa. They differ from one another in their substrate specificity; some of them seem to be metal-containing enzymes. Finally, the cathepsins must be mentioned; these are the proteolytic enzymes of spleen and other organs. Cathepsin A resembles pepsin in its specificity, whereas cathepsin B is similar to trypsin, and cathepsin C is similar to chymotrypsin.[17] Some of the cathepsins are exopeptidases, acting as aminopeptidases or carboxypeptidases.

Heretofore, we have discussed only the hydrolytic action of the proteolytic enzymes. It must not be forgotten, however, that hydrolysis is a reversible reaction and that synthesis results under suitable conditions. The equilibrium between a peptide and the products of its hydrolysis can be represented by EQUATION 1.

$$R.CO.NH.R' + H_2O \rightleftharpoons R.COOH + NH_2.R' \qquad (1)$$

K, the equilibrium constant, is equal to $[R.COOH] \times [NH_2.R']/[R.CO.NH.R'] \times [H_2O]$, where the brackets indicate molar concentrations of the four types of molecules involved. Obviously, the equilibrium can be shifted to the left-hand side of EQUATION 1 by removing water from the system or by eliminating the peptide R.CO.NH.R'. The reaction will then proceed from the right- to the left-hand side; synthesis will result in the presence of a suitable enzyme when the concentrations of R.COOH and $R'.NH_2$ are very high, the concentration of water very low, and when R.CO.NH.R' is insoluble and, therefore, removed from the system. In EQUATION 1, the two products of hydrolysis are not represented by true amino acids, which are ampholytes of the formula $\overset{+}{N}H_3.R.CO\overset{-}{O}$, but by less ionized compounds. Indeed, ionization affects the equilibrium unfavorably, shifting it far to the right-hand side of EQUATION 1. If we intend to form a peptide from two amino acids, we substitute the free amino group of one of the two amino acids and the carboxyl group of the other amino acid and in this manner decrease the electrolytic dissociation of the free carboxyl and amino groups. Benzoyl or carbobenzoxy groups are frequently used for the substitution of amino groups, whereas the carboxyl groups are substituted by esterification or by combination with ammonia or aniline. Thus, benzoyltyrosine and glycine anilide, in the presence of chymotrypsin, will yield significant amounts of the peptide benzoyltyrosylglycine anilide. Similarly, incubation of concentrated solutions of amino acid esters with chymotrypsin results in the formation of polypeptides.[18]

What is the mechanism of these syntheses? Does the proteolytic enzyme activate the carboxyl group, or the amino group, or both? It seems that all proteolytic enzymes combine with and activate the carboxyl group. The amino group can be replaced by an alcohol residue, by hydroxylamine, or by other groups quite different from amino groups. The carboxyl group, on the other hand, cannot be replaced as easily by other types of groups. We must conclude, therefore, that the proteolytic enzymes act by transferring acyl groups from one residue to another. Pepsin, trypsin, and chymotrypsin differ from each other mainly by the type of acyl residues with which they can combine and which they transfer. Chymotrypsin preferentially transfers acyl groups that contain aromatic rings; trypsin transfers acyl groups formed by a basic amino acid. Evidently, the shape and the charge of the acyl groups is decisive for the specificity of the various proteolytic enzymes. The activation of the carboxyl groups is demonstrated very impressively by REACTION 2, which is catalyzed by chymotrypsin (R = carbobenzoxy residue).[19]

$$C_6H_5.CH_2.CHNHR.COOH + H_2O^{18} \rightleftharpoons C_6H_5.CH_2.CHNHR.CO^{18}OH + H_2O \qquad (2)$$

In this reaction, chymotrypsin catalyzes the exchange of the oxygen of phenylalanine with the oxygen of water containing the isotope O^{18}. In the absence of the enzyme, no exchange takes place. In the presence of the enzyme, the carboxyl group of phenylalanine is activated and the residue $C_6H_5.CHNH_2.\overset{+}{C}O$ transferred to a hydroxyl ion, so that the heavy-oxygen isotope is built into the phenylalanine molecule. Similarly, carboxypeptidase catalyzes the exchange of the terminal glycine residue of carbobenzoxy-glycyl-glycine with free glycine containing N^{15} in its amino group. In this reaction, the carbobenzoxy-glycyl residue is transferred from glycine to N^{15}-glycine.[20] Although we cannot exclude the possibility that some of the proteolytic enzymes act by activating the amino group, this has not yet been observed. All of them seem to activate the carboxyl groups of amino acids or peptides.

The last two examples show clearly that hydrolysis is no more than a special case of a more general transfer reaction, namely, transfer of an amino acyl or peptide residue to the hydroxyl ions of water. Obviously, we should like to know whether the proteolytic enzymes give rise to such transfer reactions in the living organism. The conditions for synthesis are most unfavorable in the gastrointestinal tract where the products of hydrolysis are continually removed by absorption so that the equilibrium is continually shifted to the right-hand side of EQUATION 1, toward further hydrolysis. In the tissues, the situation may be quite different. It has been known for some time that pepsin or pancreatin can catalyze the formation of proteinlike substances from peptide mixtures. These synthetic products have been called plasteins. They are easily obtained when a proteolytic digest of ovalbumin, casein, or some other protein is neutralized, concentrated, and inoculated by a small amount of pepsin or chymotrypsin.[21] Concentration is important because it removes water and thereby shifts the equilibrium toward synthesis.

In the last two or three years we have investigated the mechanism of *plastein formation*, using radioactive amino acids. When the peptic digest of ovalbumin was incubated with chymotrypsin and traces of free radioactive phenylalanine, no significant incorporation of phenylalanine into the plastein was observed. However, when the radioactive phenylalanine was replaced by its ethyl ester, large amounts were incorporated into plastein.[22] Evidently transpeptidation had taken place. The amino acid analysis of plastein revealed that it contains much more of the nonpolar amino acids than ovalbumin, but that the content of aspartic acid, glutamic acid, and of the hydroxyamino acids is lower. The percentage of basic amino acids is approximately the same in plastein as in ovalbumin. All our observations indicate that plastein formation is, essentially, formation of large peptides at the expense of small peptides. The total number of amino and carboxyl groups seems to remain unchanged. Since this transpeptidation takes place easily under physiological conditions, it may also take place *in vivo*. It cannot be responsible for the formation of proteins from free amino acids, but it may be involved in other metabolic reactions, such as the conversion of one type of protein into another.

Finally, I should like to discuss briefly a phenomenon that may be of great importance for the clinical application of the proteolytic enzymes. It is the great resistance to proteolysis of many of the proteins in their native state, and the much higher susceptibility to proteolysis of the same proteins after denaturation. As far as we know, denaturation involves a more or less drastic change of the conformation of the peptide chains. Their mode of folding is altered. Some of the groups that, in the native protein, are buried in the interior of the globular molecules may emerge after denaturation and may thereby form points of attachment for and of attack by the proteolytic enzymes. The resistance of native proteins to digestion is easily observed when trypsin or papain act on native serum albumin, on whole serum, or on other native proteins.[23] While very little hydrolysis takes place in these homogeneous systems, rapid hydrolysis is observed after denaturation by heat or by other denaturing agents. Similarly, native collagen is resistant to trypsin, whereas its denaturation product, gelatin, is split rapidly. This resistance of native proteins to proteolytic enzymes may be responsible for the resistance of cells to self-digestion. It may also enable the organism to remove denatured proteins from degenerated or necrotic cells. The other papers in this monograph may shed more light on this important problem.

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Discussion of the Paper

H. Neurath (*Department of Biochemistry, University of Washington, Seattle, Wash.*): To the biologically minded investigator it must seem strange that some of these proteolytic enzymes are obtained by putting the freshly collected pancreas into 0.25 *N* sulfuric acid. This is far removed from physiological conditions, but it is the condition under which crystalline preparations are made. The point I wish to make is that in the protein field crystallinity is an esthetic achievement, but not necessarily an indication of purity. It follows from this that there is no assurance that the product that we are studying is really the product that exists in the pancreas.

As a matter of fact, we have some preliminary evidence to indicate that in freshly collected pancreatic juice, the secretion of the gland itself, trypsinogen and chymotrypsinogen may have a different isoelectric point than do the corresponding crystalline preparations. I think that this should be borne in mind, as it may indicate that other protein products that we are trying to prepare and characterize may have been altered during the process of isolation.

F. Haurowitz: I agree with this point of view.

SOME CONSIDERATIONS OF THE MULTIPLE SPECIFICITY OF PROTEOLYTIC ENZYMES

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The proteolytic enzymes of the extracellular secretions, particularly those of the pancreas and the gastric mucosa, have occupied a time-honored position in studies designed to elucidate the relation between the chemical structure and the enzymatic activity of these proteins. Among these, trypsin, chymotrypsin, carboxypeptidase, and pepsin have been particularly favored, since they are readily available in crystalline form and are well characterized with respect to some of their more important physical and chemical properties. These enzymes have elicited added interest since they are secreted by the cells of origin in an inactive precursor form that can be activated *in vitro* by an enzymatic process of limited proteolysis. These latter reactions, as exemplified by the activation of trypsinogen, chymotrypsinogen, or procarboxypeptidase, raise the important question as to whether the enzymatically active site is pre-existent in the precursor molecule or whether it is formed during activation by configurational rearrangements*.

Each of the crystalline proteolytic enzymes appears to contain only one active site per molecule. This site exhibits multiple specificity, as the same enzyme may be capable of catalyzing the hydrolysis of –C—N– bonds in proteins, peptides, hydroxamides, and hydrazides, of -C—O— bonds and even of –C—C– bonds, provided that the structural environment of the bond being hydrolyzed meets the specificity requirements of the enzyme. The stoichiometric reaction of some proteolytic enzymes with certain phosphorylating and acylating agents has opened new avenues of approach in the study of the mechanism of action of these enzymes and of the chemical nature of the site responsible for catalytic functions.

The advances that have been made in the study of all of these problems have been so rapid during the last few years that it will not be possible to do justice to the subject within the space and time allotted to the present contribution. It is hoped, however, that the following discussion will serve to focus attention on the more important and pressing problems in this field.

Specificity of Proteolytic Enzymes

The substrate specificity of proteolytic enzymes has been reviewed on several recent occasions,[1-3] and no attempt will be made herein to discuss in detail the structural requirements of specific substrates for the various types of proteolytic enzymes. All of them, of course, are capable of hydrolyzing peptide bonds in protein or peptide substrates, but the structural involvement of the bond being hydrolyzed is one of the features that differentiates one proteolytic enzyme from another. The nature of the configuration required for rapid hydrolysis of the peptide bond has been elucidated in the case of several enzymes after it was shown by Bergmann and his co-workers[5] that synthetic peptides of relatively simple structure can serve as substrates for many proteolytic enzymes. Quantitative kinetic studies of the rates of hydrolysis of these compounds, carried out during the last decade in several laboratories,[1, 2, 3, 6] have provided a wealth of experimental data from which certain conclusions could be drawn concerning the specificity requirements of several enzymes, including trypsin, chymotrypsin, papain,[7] pepsin,[8] carboxypeptidase, and several aminopeptidases.[4, 9, 10] Representative specific substrates for some of these enzymes are given in TABLE 1.

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* A more detailed consideration of these activation reactions is in preparation and will be published elsewhere (H. Neurath, *Advances in Protein Chemistry*, in press)

It seems now well established that the specificity requirements are absolute for certain structural components of specific substrates; that is to say, in their absence a proteolytic enzyme will have no catalytic effect on the hydrolysis of the peptide bond involved. Such absolute requirements include, in the case of trypsin, a positive charge on the side chain of the amino acid contributing the carboxyl group to the peptide bond or, in the

TABLE 1
REPRESENTATIVE SUBSTRATES FOR PROTEOLYTIC ENZYMES[1-4]

Enzyme	Substrate			
	R_1	R_2	↑	R_3
Trypsin	Benzoyl	L-arginine		amide
	Benzoyl	L-arginine		ester
	p-Toluenesulfonyl	L-arginine		ester
	Benzoyl	L-lysine		amide
		ethyl(8-amino-3-keto)octanoate		
Chymotrypsin	Acetyl	L-tyrosine		amide
	Acetyl	L-tryptophan		amide
	Acetyl	L-phenylalanine		amide
	Nicotinyl	L-tryptophan		amide
	Acetyl	L-hexahydrophenylalanine		amide
	Acetyl	L-tyrosine		ethyl ester
	Acetyl	L-tyrosine		hydroxamide
		5-(*p*-hydroxyphenyl)-3-ketovalerate		
Pepsin	Acetyl	L-phenylalanyl		L-phenylalanine
	Acetyl	L-tyrosyl		L-tyrosine
	Carbobenzoxyl	L-glutamyl		L-tyrosine
	Glycyl	L-tyrosyl		L-tyrosine
Papain	Benzoyl	L-arginine		amide
	Toluenesulfonyl	L-arginine		methyl ester
Carboxypeptidase	Carbobenzoxyl	glycyl		L-phenylalanine
	Carbobenzoxyl	glycyl		L-tyrosine
	Carbobenzoxyl	glycyl		L-tryptophan
	Benzoyl	glycyl		β-phenyllactate
		Chloroacetyl		L-phenylalanine

case of carboxypeptidase, the presence of an α-carboxyl group on the amino acid that contributes the amino group to the susceptible peptide bond (TABLE 1). In all cases, hydrolysis is dependent on the optical configuration of one or more elements of the peptide.[11]

Other structural components of specific substrates may have a less profound influence on the catalytic process, in that their removal or chemical modification retards, but does not abolish, the enzymatic process. Included therein are the (secondary) peptide group, adjacent to the one being hydrolyzed; the presence of a cyclic, preferably aromatic, structure in the side chain of specific substrates for chymotrypsin[2, 10, 12] and carboxypeptidase;[2, 13] or the presence of two adjacent aromatic side chains in specific substrates for pepsin.[8]

The spectrum of substrates for proteolytic enzymes was considerably enlarged when it was found that the hydrolyzable peptide bond could also be replaced by other bonds, provided the remaining structural characteristics of the substrate remained intact. The first major advance in this direction was the demonstration that trypsin,[14] chymotrypsin,[15] and carboxypeptidase[16] were capable of hydrolyzing compounds in which the peptide bond was replaced by an ester bond, these esters being hydrolyzed much more rapidly than the corresponding amides or peptides. Thus, in the case of trypsin and chymotrypsin, replacement of the —C—N— bond by a —C—O— bond increased hydrolysis rates by two or three orders of magnitude. It is worthy of note, however, that this does not seem to be a general property of all proteolytic enzymes, as certain of the aminopeptidases have been found to be devoid of esterase activity.[13, 17]

Other bonds susceptible to hydrolysis by certain proteolytic enzymes, such as chymotrypsin, include the —C—N— bond of hydrazides[18] and hydroxamic acids,[19] the —C—S— bonds of thio esters[20] and, as recently shown, the —C—C— bond of certain keto acids. Thus, Doherty[21] was able to demonstrate that the keto ester, ethyl 5-(*p*-hydroxyphenyl)-3-ketovalerate, was hydrolyzed by α-chymotrypsin to yield *p*-hydroxyphenylpropionic acid under the same conditions under which the enzyme is active toward specific peptide or ester substrates. The site of hydrolysis in this compound, whose structure is given in FIGURE 1, is the 2-3 carbon-carbon bond rather than the terminal one, since the free acid, 5-(*p*-hydroxyphenyl)-3-ketovaleric acid, is also hydrolyzed, although, in keeping with the structural requirements of specific substrates for chymotrypsin, at a lower rate. While the metabolic significance of the hydrolytic scission of a carbon-carbon bond by a proteolytic enzyme such as chymotrypsin remains obscure, the phenomenon itself is of considerable importance, as it extends the specificity range of this enzyme to a new type of compound. More recently, a similar structural analogue of specific substrates for trypsin also has been described, that is, ethyl(8-amino-3-keto)octanoate, wherein the carbon-carbon bond in positions 2 and 3 is likewise hydrolyzed.[22]

The question may be raised, of course, whether the same enzyme is endowed with multiple functions or whether the catalytic effect on such a variety of chemical structures is due to the presence of more than one enzyme

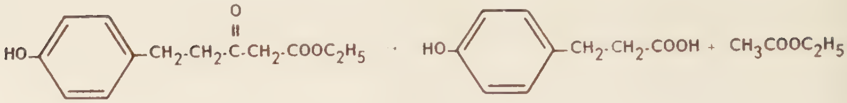

FIGURE 1

or more than one enzymatic site in the enzyme preparation. This problem must be solved for each enzyme in question by ascertaining that one or more of the following criteria are applicable:

(1) The ratio of the specific activities of an enzyme toward two or more substrates should remain constant throughout the purification process; (2) this ratio should also be unaffected by partial inactivation due to denaturation or *p*H changes; (3) the addition of a specific inhibitor should affect both activities in a manner predicted by the kinetic equation for competitive inhibition; (4) the hydrolysis of a mixture of two substrates by the same enzyme should be accountable kinetically on the assumption that both substrates have reacted with the same active site.[23] In the case of the enzymes that have been considered here, it seems reasonably certain that the multiple enzymatic functions are all mediated by a single site of a single enzyme.

The Specificity of Proteolytic Enzymes Toward Protein Substrates

Recent refinements of experimental methods have paved the way for determining the specificity requirements of proteolytic enzymes toward their natural substrates, that is, the proteins. Reference is made in particular to the various methods of end-group analysis that permit the identification of the new N- and C-terminal groups that are formed upon hydrolysis of a peptide bond. As these methods have been recently reviewed by several authors,[24-27] it will suffice, for the present discussion, simply to refer to the most common ones now in use, which include

(1) For N-terminal groups: (a) the fluorodinitrobenzene (FDNB) method of Sanger; (b) the phenylthiohydantoin method of Edman and Fraenkel-Conrat; and (c) the enzymatic method of Smith.[28] The latter is based on the liberation of N-terminal amino acids in a sequential manner under the influence of a purified kidney aminopeptidase.

(2) Only two methods for determining C-terminal groups are in general use at the present time: (a) the chemical hydrazinolysis method of Akabori[26, 27]; and (b) an enzymatic method based on the sequential liberation of C-terminal amino acids under the influence of carboxypeptidase.[25, 29] Neither of these procedures is entirely satisfactory; the chemical method involves considerable destruction of some of the amino acid being determined, particularly arginine, and the enzymatic method is restricted to the liberation of those amino acids that conform to the specificity requirements of the enzyme, for C-terminal arginine and lysine are hydrolyzed only slowly, if at all, and C-terminal proline is entirely resistant to the action of this enzyme.

Further experimental advance in the study of the action of proteolytic enzymes on protein substrates was made by the introduction of the *p*H-stat of Jacobsen and Léonis,[30] which proved to be particularly valuable for determining the number of hydrolyzed peptide bonds.[31]

Trypsin. It can be stated with considerable confidence that the specificity requirements of crystalline trypsin, as deduced from studies on synthetic substrates, apply equally to the action of this enzyme on proteins (TABLE 2). This was first established by the work of Sanger and his co-workers in which crystalline trypsin was used to fragment the B-chain of oxidized insulin into shorter peptides. In this case, only the arginyl-glycyl bond (positions 22-23) and the terminal lysyl-alanine bond (positions 29-30) were hydrolyzed.[32] In the tryptic hydrolysis of β-corticotropin the sites of cleavage were, similarly, lysyl-valyl, arginyl-tryptophanyl, lysyl-lysyl, lysyl-aringyl, and arginyl-arginyl bonds.[33] Other examples include the tryptic hydrolysis of oxidized ribonuclease,[34] yielding a series of peptides that contain exclusively lysine or arginine as C-terminal groups, and similar types of peptides from tryptic digests of glucagon.[35]

Chymotrypsin. Although the specificity spectrum of chymotrypsin is wider than that of trypsin, it may be said that generally this enzyme splits in proteins precisely the bonds that one would expect from studies on specific substrates (TABLE 2). This is true of the action of chymotrypsin on the

TABLE 2
PEPTIDE BONDS HYDROLYZED BY THE ACTION OF PROTEOLYTIC ENZYMES ON PROTEIN OR PEPTIDE SUBSTRATES OF KNOWN AMINO ACID SEQUENCES

Enzyme	Substrate	Bonds hydrolyzed*
Trypsin	Insulin (oxidized B chain)[32]	-Arg-Gly; -Lys-Ala-.
	β-Corticotropin[33]	-Arg-Try-; -Lys-Lys-; -Lys-Val-; (-Lys-Arg-); (-Arg-Arg-).
	Ribonuclease (oxidized)[34]	-Lys-Phe-; -Lys-Ser-; -Arg-Asp(NH_2)-; -Lys-Asp-; -Lys-Asp(NH_2)-; -Lys-His-;
	Melanophore-stimulating hormone[38]	-Lys-Met-; -Arg-Try-;
Chymotrypsin	Insulin (oxidized B chain)[32]	-Tyr-Leu-; -Phe-Tyr-; -Tyr-Thr-; (-Leu-Tyr-).
	β-Corticotropin[33]	-Tyr-Ser-; -Phe-Arg-; -Try-Gly-; (-Lys-Lys-)†; -Leu-Gly-;
	Ribonuclease (oxidized)[37]	-Phe-Glu(NH_2)-; -Leu-Thr-; -Tyr-Glu(NH_2)-; -Tyr-Lys-;
	Melanophore-stimulating hormone[38]	-Tyr-Lys-; -Phe-Arg-; -Try-Gly-.
Pepsin	Insulin (oxidized B chain)[32]	(-Phe-Val-); (-Glu(NH_2)-His-); -Leu-Val-; (-Glu-Ala-); (-Ala-Leu-); (-Leu-Tyr-); -Tyr-Leu-; (-Gly-Phe-); -Phe-Phe-; -Phe-Tyr-.
	β-Corticotropin[33]	-Glu-Asp-; -Glu(NH_2)-Leu-; -Leu-Ala-; -Phe-Pro-.

* Bonds in parentheses indicate minor points of cleavage.

† Probably due to a trypsin-like contamination in chymotrypsin preparations used.

A and B chains of oxidized insulin,[32] on α- and β-corticotropin,[33, 36] on oxidized ribonuclease,[37] on glucagon,[35] and on the melanophore-stimulating hormone.[38] In all these cases, tyrosyl, phenylalanyl, or tryptophanyl bonds are the primary sites of attack.

Pepsin. The specificity requirements of crystalline pepsin are not fully elucidated as yet. The significance of studies on synthetic substrates is partly obscured by the fact that many of these are maximally hydrolyzed at *p*H 4, whereas the *p*H optimum for the action of this enzyme on protein substrates is lower (*p*H 2 or less).[1, 4] It is possible that these discrepancies are due in part to the presence in crystalline preparations of pepsin of a catheptic impurity that has a higher *p*H optimum.[1] However, more recent studies by Baker[8] seem to indicate that peptide bonds between two adjacent aromatic side chains are split most readily by crystalline pepsin at *p*H 2. However (TABLE 2), studies on peptides liberated by pepsin from protein chains of known amino acid sequence[32, 33, 39] indicate a broader spectrum of specificities.

Carboxypeptidase. The action of carboxypeptidase on protein and peptide substrates has been reviewed recently,[1, 25, 29] and all available data seem to indicate that the specificity requirements as deduced from the action on simple dipeptides or tripeptides are also observed when longer peptides or proteins are used as substrates. However, as in the case of endopeptidases, the secondary or tertiary structure of the protein substrate may offer resistance to enzymatic hydrolysis, as evidenced by the finding that, in some cases, carboxypeptidase may be active only after the protein has been denatured by concentrated urea solutions, wherein the exopeptidase remains active, at least for limited periods of time.[40]

In this connection, it might be well to draw attention to some factors that may not only obscure studies of the substrate specificity of proteolytic enzymes but may, in fact, lead to misleading interpretations. One of these is the presence of adventitious impurities of other proteolytic enzymes in crystalline preparations of the enzyme being studied. This situation is exemplified by the frequent presence of chymotrypsin in crystalline trypsinogen and trypsin, and by the presence of either of these enzymes, or of other endopeptidases, in crystalline carboxypeptidase.[25, 29, 41] The contributions of these adventitious impurities may be minimized by the addition of specific inhibitors of the contaminating enzyme. Diisopropylphosphofluoridate (DFP) is among the most frequently used inhibitors of chymotrypsin and trypsin. Another factor to be borne in mind is the fact that in a sequential reaction involving the hydrolysis of several peptide bonds,[42a] the nature of the product formed in the first step may have a modifying influence on the second and subsequent steps*. This may be illustrated best by consideration of the action of pepsin on the A and B chains of oxidized insulin, in which, for instance, a leucyl-valyl bond in the sequence histidyl-leucyl-valyl in one part of the B chain is rapidly split, whereas a similar bond in the sequence tyrosyl-leucyl-valyl, occurring in a different portion of the chain, is resistant.[1, 32] Since pepsin readily attacks the tyrosyl-leucyl bond, the free amino group of the leucyl residue formed may prevent the subsequent hydrolysis of the leucyl-valine bond. In the former case, the histidyl-leucine bond is not hydrolyzed, thus rendering the leucyl-valine bond susceptible to attack.

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* For a discussion of the kinetic aspects of stepwise proteolysis, see Linderstrøm-Lang.[42a]

Transpeptidation reactions, catalyzed by the very enzymes that are employed for proteolytic degradation, have never been fully excluded as a disturbing factor in sequential amino acid analysis. Trypsin and chymotrypsin are both known to catalyze transpeptidation[42b] of the type

$$\mathrm{RCONHR'} + \mathrm{R''COOH} \rightleftharpoons \mathrm{R''CONHR'} + \mathrm{RCOOH}$$

and, although in all the cases considered, a unique amino acid sequence has been established from data derived from enzymatic and acid degradation of proteins, this problem deserves more attention in future work.

The Reaction of Proteolytic Enzymes with Phosphorylating and Acylating Agents

Several of the proteolytic enzymes react stoichiometrically with a number of alkoxyphosphates, such as diisopropylphosphofluoridate (DFP), giving rise to inactive products containing one mole of phosphorus per mole of enzyme protein.[43, 44] These reactions do not occur when the enzymes are in the zymogen form, nor after denaturation, and the *p*H dependence of phosphorylation follows closely the *p*H dependence of the activities of these enzymes toward natural or synthetic substrates. It was suggested, therefore, that the reaction of proteolytic enzymes with DFP and its derivatives is enzymatic in nature, the only difference being that one of the reaction products, that is, the alkoxyphosphate, is irreversibly bound to the enzyme, thus causing inactivation and precluding enzyme turnover.[45] Chymotrypsin, trypsin, papain, and several other proteolytic enzymes have been found to be inactivated by these organic phosphorylating agents. However, other proteolytic enzymes, such as carboxypeptidase or some of the aminopeptidases, seem to be unaffected by the alkoxyphosphates.

There are other types of organic reagents that, similarly to DFP, seem to react stoichiometrically with proteolytic enzymes and cause their inactivation. These include the fluorescent dye 1-dimethylaminonaphthalene-5-sulfonyl chloride, which is capable of interacting with chymotrypsin in integral molar proportions; a 1:1 combination is sufficient to cause complete enzyme inactivation.[46] In addition, it has been shown recently that both chymotrypsin[47-51] and trypsin[52] react stoichiometrically with such acylating agents as *p*-nitrophenylacetate, yielding, under certain conditions, a stable monoacetyl enzyme. Acylation may be achieved similarly by reaction with certain acid anhydrides and acid chlorides;[53-57] the acyl enzyme produced has been enzymatically inactive in all cases that have been studied.

It is likely that in all cases reaction of the organic reagent with the proteolytic enzyme causes the same group on the enzyme molecule to be phosphorylated or acylated. However, the various organic derivatives differ considerably in their stability. Thus, inhibition by the phosphorylating agents cannot be reversed by dilution or by dialysis and requires the action of strongly nucleophilic reagents, such as nicotinohydroxamic acid or its analogues.[44, 53] In contrast, the acetyl group of monoacetyl chymotrypsin can be transferred readily at pH 5 to hydroxylamine[49] and, at pH 7 or higher, is spontaneously hydrolyzed in water to yield active chymotrypsin.

The relative ease of hydrolysis of acyl enzymes, with the concomitant reappearance of enzymatic activity, has invited investigation of the kinetics of the acylation and deacylation reactions.[47, 50, 51, 52, 54] These reactions may be depicted formally as shown in EQUATIONS 1 and 2:

$$\mathrm{Ch} + \mathrm{AcOR} \rightarrow \mathrm{AcCh} + \mathrm{ROH} \tag{1}$$

$$\mathrm{AcCh} + \mathrm{H_2O} \rightarrow \mathrm{Ch} + \mathrm{Ac^-} + \mathrm{H_3O^+} \tag{2}$$

The acylation by p-nitrophenylacetate (NPA) can readily be measured spectrophotometrically by the appearance of p-nitrophenol. This is shown by curve 2 of FIGURE 2, which depicts the progress curve for the acetylation of chymotrypsin by a twofold molar excess of NPA at pH 5. At higher pH, the acetyl enzyme initially formed will be deacylated continuously, as shown by curve 1 of FIGURE 2, wherein the linear portion represents the steady state, in which the rate of change of concentration of the acyl enzyme remains constant until the acylating agent becomes exhausted, as indicated by the leveling off after longer periods of time. As indicated previously, acylation may occur with a variety of acid anhydrides and acid chlorides, as shown in

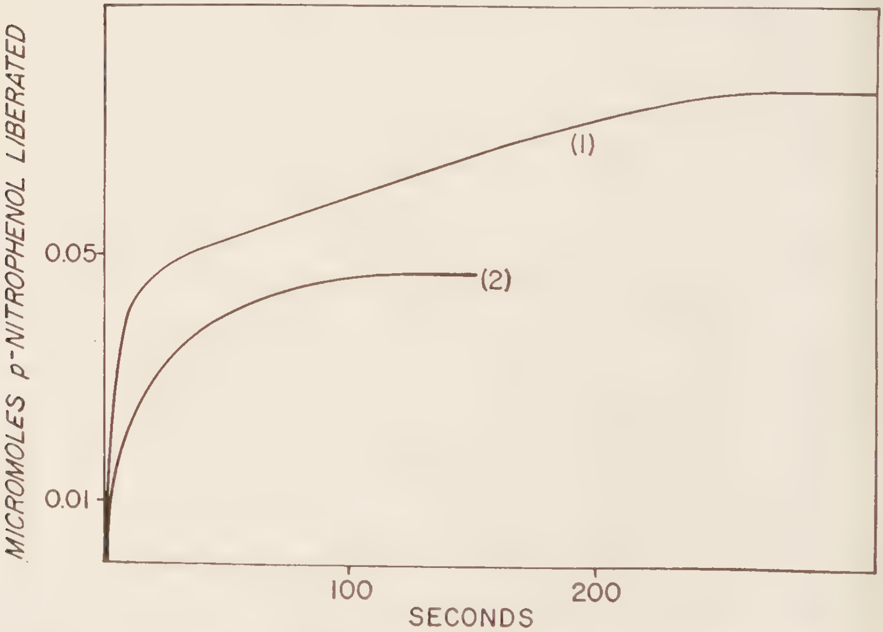

FIGURE 2. Progress curves for the reaction of δ-chymotrypsin with p-nitrophenylacetate, as recorded spectrophotometrically by the amount of p-nitrophenol liberated. Curve 1, reaction at pH 7.5; curve 2, reaction at pH 5.5. Curve 2 corresponds to the formation of monoacetyl-δ-chymotrypsin. (Data of Dixon, Dreyer, and Neurath.[51])

Table 3

Reagents Causing Acylation of the Enzymatic Site of Chymotrypsin

p-Nitrophenyl acetate
Acetic anhydride
n-Propionic anhydride
n-Butyric anhydride
n-Valeric anhydride
n-Hexanoic anhydride
n-Heptanoic anhydride
Benzoyl chloride
Phenylacetyl chloride

TABLE 3. The hydrolytic deacylation of acyl chymotrypsin can be studied conveniently by the rate of appearance of enzymatic activity, as measured by the hydrolysis of the synthetic substrate acetyl-L-tyrosine ethyl ester (ATEE). The progress of deacylation, at *p*H 7, is shown in FIGURE 3, in which the base uptake at constant *p*H, resulting from the hydrolysis of ATEE, as recorded automatically in a *p*H-stat, is plotted as a function of time. The deviation from zero-order kinetics, observed in the early phase of the reaction, is due to the deacylation of the enzyme and can be described satisfactorily by first-order reaction kinetics.

Nature of the catalytic site. One of the most intriguing questions relating to the reaction of proteolytic enzymes with organic phosphorylating and acylating agents is the identification of the group involved in these inter-

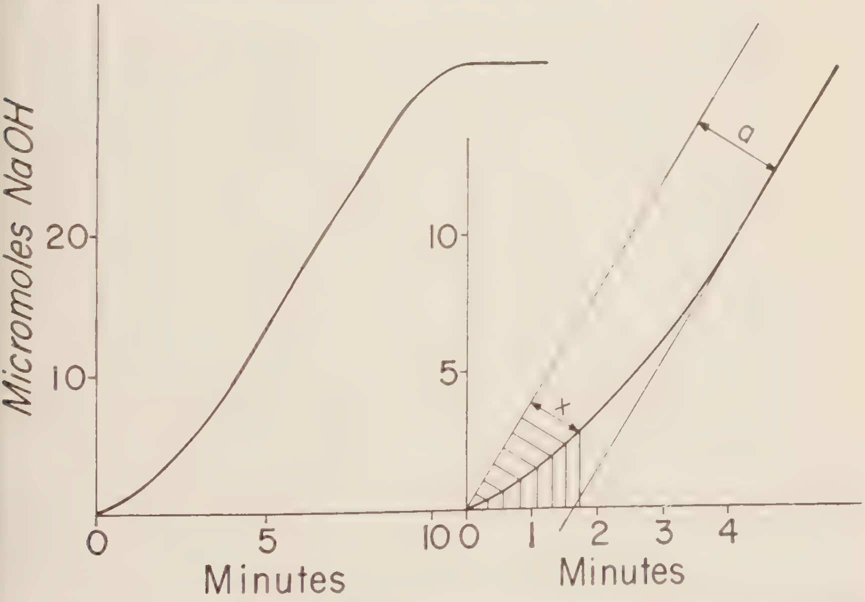

Figure 3. Measurements of the rate of deacetylation of monoacetyl δ-chymotrypsin, as determined by the rate of hydrolysis of the chymotrypsin substrate, acetyl-L-tyrosine ethyl ester, by the free enzyme. The first-order rate constant for the deacetylation was measured by the graphic method illustrated in the inset. (Data of Dixon and Neurath.[2])

actions. Since, in all cases studied, a 1:1 combination results in enzyme inactivation, it is likely that the group affected by this combination is part of the catalytic site.

Degradation studies on the inactive diisopropylphosphoryl (DIP) derivatives of chymotrypsin, trypsin, and cholinesterase have shown in each case that the hydroxyl group of serine is phosphorylated.[44, 55, 56] However, it has been suggested that this is not the initial focus of interaction between the phosphorylating agent and the enzyme, and various lines of evidence have been adduced in support of the view that the imidazole side chain of histidine is involved in the catalytic function of the enzymes and in their interaction with organic phosphates.[44, 54] In brief, the supporting evidence is: (1) the *p*H dependence of inhibition by DFP, as the *p*H dependence of enzymatic activity, can be described by the dissociation curve of an acid with a *p*K of 5 to 7;[57] (2) of all the amino acids tested, histidine and tyrosine were uniquely able to catalyze the hydrolysis of DFP, indicating the formation of unstable DIP-amino acid complexes as intermediates; serine was inactive under these conditions;[58] (3) histidine derivatives, as well as free imidazole or *N*-methyl imidazole, have been found to be effective in catalyzing the hydrolysis of NPA;[59, 60] (4) photo-oxidation of chymotrypsin destroys, among others, histidine side chains, and it abolishes reaction with DFP.[61]

If the imidazole group of a histidine side chain were in fact the primary site of stoichiometric phosphorylation or acylation, it would have to be assumed that the complex initially formed is unstable and that the organic substituents are subsequently shifted to a strategically located serine residue. The maintenance of a serine group in such a favorable position could be the result of (1) the two residues being close to each other in the polypeptide sequence or (2) the residues being brought into juxtaposition by their presence in adjacent turns of an α-helix or by the close apposition of two separate parts of an α-helix (or two separate α-helices).[63] However, the study of serine peptides resulting from the degradation of DIP-chymotrypsin or DIP-trypsin thus far has not revealed histidine as a constituent, so that the first of these interpretations is unlikely.[56, 62, 63, 64] In the case of DIP-chymotrypsin, the composition of the DIP-peptide, obtained by enzymatic hydrolysis, was Gly.Asp.Ser.Gly.[Pro.Leu(Gly)]*. In the case of DIP-trypsin, the DIP-peptide, obtained by chymotryptic hydrolysis, contained Cys, Asp, Glu. Gly, Ser, Ala, Pro, Val, and Lys. Lysine probably occupies the C-terminal position and cystine the N-terminal position.[63]

The possibility that the postulated histidine and serine residues are brought into juxtaposition by folding of the polypeptide chain was studied in experiments in which freshly prepared DIP^{32}-trypsin was denatured in 8 *M* urea in order to interfere with group transfer by chain separation. However, no release of the radioactive label from the presumably unstable primary site into the solvent was observed; nor was DFP taken up if the protein was first denatured in 8 *M* urea.[65] Moreover, no DFP^{32} uptake occurred when DIP-trypsin, allowed to stand long enough for the shift to the stable site to have been completed, was allowed to react with freshly added DFP.[32] Additional evidence against the stoichiometric acetylation of a histidine side chain was provided by experiments designed to detect the characteristic ultraviolet absorption, at 245 mμ, of acetyl histidine in freshly prepared acetyl chymotrypsin; no such spectral characteristic was observed.[51] These experiments do not preclude the possibility, however, that a short-lived DIP-histidyl derivative catalyzes the reaction of phosphorylating or acylating agents with a serine residue. Both the acetylation and deacetylation of chymotrypsin are dependent on the structural integrity of the protein as a whole. Thus, in the presence of 8 *M* urea, no acetylation by NPA occurs,[51, 66] whereas the reactivity of the acetyl group in acetyl chymotrypsin toward hydroxylamine, at *p*H 5.5, is abolished by denaturation in 8 *M* urea (at *p*H 3.0). However, when denaturation is reversed, deacetylation by hydroxylamine, or by water at *p*H 7.5, is restored.[51] These observations indicate clearly that the properties of the specially reactive group in these enzymes cannot be interpreted readily in terms of the known properties of any amino acid side chain in isolation, as the reactivity of the group in question is functionally related to the specific structure of the native protein. The indirect participation of an ionizing group with a *p*K of 6 to 7 is indicated by the observation that both the acetylation and deacetylation[51, 52, 54] are *p*H-dependent; the *p*H dependence of deacetylation of acetyl chymotrypsin is similar to the *p*H dependence of the hydrolysis of the synthetic substrate ATEE by free chymotrypsin.

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* The dot between constituents denotes sequential order, whereas the relative position of the amino acids given in parentheses and brackets remains to be elucidated.

The study of the chemical structure of larger peptide fragments derived from the enzymatic digestion of DIP-chymotrypsin, DIP-trypsin, and C^{14} acetyl-trypsin, now in progress in this laboratory and elsewhere, should provide much important information on the chemical configuration of the site responsible for the reaction with phosphorylating and acylating agents, and for catalytic functions.

Lastly, we may raise the question as to whether the entire structure represented by the molecule of a proteolytic enzyme is essential for enzymatic activity and, if not, how far degradation can occur before enzymatic activity ceases. It is unlikely that we shall find an answer that is applicable to all proteolytic enzymes, as the molecular entities represented by such enzymes as papain, trypsin, chymotrypsin, and carboxypeptidase differ greatly from one another in structural details. It is worthy of note, however, that in the case of papain it has been possible to carry out sequential degradation of the enzyme, by means of highly purified aminopeptidase, to the extent of removing two thirds of the mass of the molecule without impairing the catalytic properties.[28] It is clear, therefore, that in this particular instance the catalytic site is unsymmetrically disposed within the molecule, and that it is probably located near the C-terminal region of the constituent polypeptide chain. There are preliminary observations suggesting that dialyzable fragments of unspecified size, resulting from the autodigestion of pepsin and other proteolytic enzymes, possess enzymatic activity,[67] and the question of the "active fragment" is uppermost in the minds of investigators in this field.

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Discussion of the Paper

F. Haurowitz (*Department of Chemistry, Indiana University, Bloomington, Ind.*): In the discussion of specific activity of chymotrypsin you did not mention methionine or threonine esters or peptides. Were they not used for synthesis?

H. Neurath: Methionyl and threonyl peptides are hydrolyzed by chymotrypsin, but slowly as compared to others that I have mentioned.

F. Haurowitz: Do you think there is any reason to assume that the active group of the enzyme contains an accumulation of either positively or negatively charged groups? You know that one of the amazing findings of sequential analysis in the last few years has been that so many glutamyl and aspartyl residues are in one place and so many histidyls and arginyls are in others, just as in your example.

H. Neurath: I think that there is no question that in the case of trypsin there must be a negatively charged group near the active site that attracts the positively charged side chains of lysine or arginine. I would not necessarily make the same assumption for chymotrypsin, but I think one should be open-minded and consider this as a possibility.

M. M. Rapport (*Sloan-Kettering Institute for Cancer Research, New York, N. Y.*): Do you believe that acylation of the hydroxyl group could be one of the biological mechanisms for inactivation of enzymes?

H. Neurath: I think that it is within the realm of possibility because acyl transfers do occur in metabolic pathways, and if these occur in the cells of origin of proteolytic enzymes, the possibility of their action is very real.

W. Troll (*New York University College of Medicine, New York, N. Y.*): Is the hexapeptide from trypsinogen a trypsin inhibitor?

H. Neurath: It is not; when you add it back to the trypsin it has no inhibitory functions whatsoever. I think this can be understood if we assume that the hexapeptide will not necessarily be bound by trypsin at the site that it originally occupied.

PLASMIN: THE HUMORAL PROTEASE*

By Daniel L. Kline and J. B. Fishman

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Although the title of this article is very broad, the appearance of an excellent and comprehensive review of the fibrinolytic system in blood recently published by Astrup[1] permits us here to concentrate on two aspects of this subject with which we have been particularly concerned: the chemical purification and identification of certain of the enzymes involved in this system. It will become evident that the identification of these enzymes of necessity involved a critical re-examination of the theories that have been proposed to explain the fibrinolytic systems that exist in human and animal blood.

The first real progress in the purification from human blood of the proteolytic enzyme now known as plasminogen was made in 1946 when the Cohn method of alcohol fractionation[2] was shown to yield a subfraction of the euglobulins, Fraction III, that contained plasminogen in a state of purity ten times greater than that of the original plasma. This purification was, of course, incidental to the purification of the plasma proteins in general. The first significant paper on the isolation of human plasminogen itself was that of Remmert and Cohen[3] in 1949. They reported a partial purification from serum based upon the adsorption of plasminogen upon kaolin and subsequent elution. Although this method proved to give inconsistent results in our and other laboratories, their work showed that the usual protein-purification techniques available at that time ammonium sulfate, alcohol, and isoelectric precipitation– were not helpful in regard to plasminogen because of the intense coprecipitating characteristics of this enzyme.

In 1951 Christensen and Smith[4] were the first investigators to utilize the Cohn Fraction III as a starting material, and they made the important observation that plasminogen was soluble and stable in acid solution. Work on this problem was started in our laboratory in 1950, but we made no progress until Christensen's paper appeared. By 1952 a procedure based upon an acid extraction, the selective inactivation of other proteins in Fraction III at *p*H 11, a second acid extraction, and the precipitation of plasminogen in the presence of phosphate ions was developed[5] and provided a fibrinolytic material of sufficient purity to warrant testing in animals (TABLE 1). This appears to be the only method available at present of preparing plasminogen of this purity, possessing an activity 400 times greater than that of serum. In the conversion of plasminogen to plasmin, streptokinase (SK) was selected as the activator of choice since it was available commercially as Varidase† and had been used as a clinical agent in localized treatment. Using plasminogen prepared essentially by the procedure described

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† Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

TABLE 1

THE YIELD AND PURITY OF PLASMINOGEN FROM 10 GM. OF FRACTION III

	Proteolytic units	Nitrogen (mg.)	Proteolytic units mg. N
Serum	1500		0.14
1st acid extract	1500	306.0	4.9
2nd acid extract	1042	69.2	14.8
Final product	531	9.1	59.1

above and activated with SK, Cliffton[6, 7] was the first to show, in convincin animal experiments, that a fibrinolytic agent with clinical possibilitie was now available. These results have been confirmed in several othe laboratories.[8, 9, 10]

Unfortunately, certain toxic manifestations, principally a fall in bloo pressure and a rise in body temperature that appear after the intravenou administration of Varidase,[11] also were present after the injection of SK-activated plasminogen. Accordingly, experiments were instituted to isolat active plasmin, in the hope that such a preparation would be less toxic. I was found that plasmin could be precipitated from solution by the additio of alcohol to 20 per cent by volume.[12] The results obtained are shown i TABLE 2. Varidase itself remained in solution under these conditions. Th assay method used to follow this purification involved the lysis of an impur clot and was, therefore, primarily a measure of the activator. This poin will be considered in more detail shortly. The plasmin so obtained has bee tested *in vivo* in three laboratories and has been shown to be free of an effect upon the blood pressure and to have a diminished although still significant and somewhat unpredictable pyrexic effect. A more recent preparatio that will be discussed later appears to be even less pyrogenic in human (J. L. Ambrus, personal communication).

During the time that chemical purification of plasminogen and then o plasmin was being carried out in our laboratory, investigations by othe workers, particularly Astrup and Müllertz in Denmark, and Sherry and hi group in the United States, brought to light new observations that cause them to suggest a revision of the fibrinolytic system and raised the possibility that the proteins we had purified might not be plasminogen an plasmin, but other factors in the fibrinolytic system. Our attempts t clarify this situation will be considered next.

The original fibrinolytic theory proposed simply that plasminogen is converted to plasmin by activators such as SK and chloroform. It was wel known that SK produces fibrinolytic activity when added to human plasm but is ineffective as an activator of the plasminogen of other species. Staphylokinase produces activity in dog plasma[13] and fibrinokinase in bovin plasma.[14] It was, therefore, believed that plasminogen varied from specie to species and that it would be necessary to find a specific activator for eac species, the degree of specificity, of course, being relative rather than abso

TABLE 2
ALCOHOL FRACTIONATION OF 100 MG. OF PLASMINOGEN AFTER STREPTOKINASE ACTIVATION

Per cent alc.	Nitrogen	Fibrinolytic activity	Specific activity	Total act. in fraction
	mg./ml.	units/ml. × 10^3	units/mg. N × 10^3	units × 10^4
0	0.67	40	60	100
10	0.29	123	423	60
21	0.21	44	207	20
Remainder	0.89	4	4.5	2
0	0.82	40	49	100
10	0.37	100	294	50
15	0.21	36	170	20
21	0.14	18	127	9
0	0.76	40	49	100
21	0.58	192	330	100
0	1.03	40	39	100
21	0.76	155	203	80

iute. However, both Astrup's[illegible] and Sherry's[16] groups independently discovered that human plasminogen plus SK produced a universal activating enzyme, that is, an activator of the plasminogen of any species. The explanation offered by Astrup's laboratory for this finding was that the plasminogen does not differ from one species to another, but that an additional factor, a proactivator, that is present in human plasma, but is lacking in the blood of other species, is necessary in the system.

With the observation that human plasminogen plus SK produces a universal plasminogen activator, it was soon demonstrated that the bovine fibrinogen used in clot assays contained large amounts of plasminogen and, therefore, that these assays measured not only the activity of the material being tested, but also the activity produced by the contamination present in the clot.[15, 16] It was obviously necessary to eliminate the large contribution of plasminogen inadvertently added to the test system in order to evaluate the potency of a fibrinolytic preparation.

The laboratories in Denmark and the United States attacked this problem n different ways. Astrup's group, having developed a plate method of assay n which a drop of enzyme is placed on a thin layer of fibrin and the size of the lysed area is measured, destroyed plasminogen in the fibrin by heating the plates before adding the drop of enzyme solution. Sherry, using an assay method in which the addition of enzyme preceded clot formation, was unable to heat the fibrinogen since it would then be unable to react with hrombin to form a clot. He therefore attempted to purify the fibrinogen by chemical means. In addition, assuming that proteolytic activity is equal to or proportional to fibrinolytic activity, Sherry's group used the splitting of arginine and lysine esters, which were discovered in their laboratory to be substrates of plasmin,[17] in a test system devoid of fibrinogen or thrombin. Using the techniques described above, both groups found that plasmin activity was greatly diminished when tested with reagents free of plasminogen. Sherry, who had at first believed that proactivator and plasminogen are identical,[16] accepted the view that proactivator is a separate entity when the experiments on synthetic substrates showed that two factors were produced when SK was added to plasminogen and that the activation of plasminogen was catalytic, whereas the reaction of proactivator with SK was stoichiometric.[18] The fibrinolytic system proposed by Astrup in 1954 and now generally accepted is shown in FIGURE 1. It should be kept in mind that the existence of a proactivator in human plasma was postulated to explain why the plasminogens of other species are not activated by SK alone, but require the presence of human plasma. Proactivator has not as yet been isolated, purified, or even demonstrated to be an entity, except by indirect inference.

This was the situation when we began to investigate whether we had purified proactivator and activator or plasminogen and plasmin from human Fraction III. We avoided the use of heated fibrin plates when we found that the susceptibility of heated fibrin to fibrinolytic activity was greatly reduced. Analysis of the data of Müllertz[15] also showed that heating did more than destroy the plasminogen in the fibrin clot since the addition of fresh plasminogen failed to restore the activity to more than 20 per cent of the preheated level in his experiments. Attempts to purify fibrinogen by

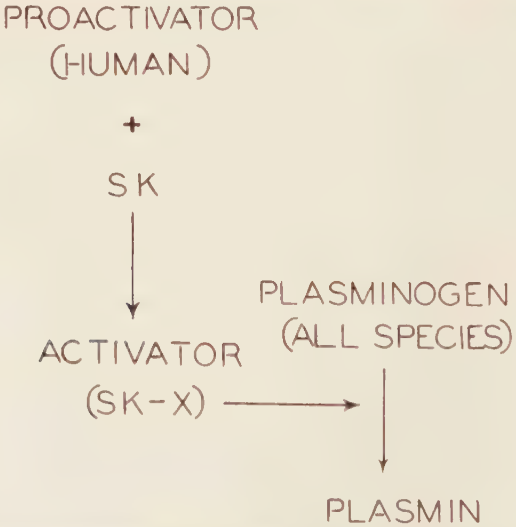

FIGURE 1. Current fibrinolytic scheme. SK reacts stoichiometrically with proactivato to form activator. The activator catalyzes the conversion of plasminogen to plasmin.

the method of Laki,[19] as recommended by Sherry, were unsuccessful in our hands. The reason for this is not yet clear, but we have obtained evidence that the thrombin we used contained plasminogen, which confused the situation. Moreover, SK has a markedly inhibitory effect in clots made with purified fibrinogen so that, unless the optimal concentration of SK is used, there may be an apparent but unreal decrease in the fibrinolytic activity of the enzyme preparation being tested. The use of synthetic substrates was also avoided because there is no definite proof that the splitting of amino acid esters is equivalent to the dissolving of a clot. There is considerable evidence to show that fibrin is a complex in which the fibrils are held together by many forces other than peptide bonds.[20] The dissolving of such a gel n all probability involves, not only proteolysis, but also the splitting of non-peptide linkages. In our laboratory we have obtained evidence that the proteolysis of casein and the lysis of a clot that is free of contaminants do not parallel each other under certain conditions (H. A. Newman, unpublished).

We therefore attempted to produce a clot made of ingredients that were ree of plasminogen. In trying to evaluate the source and amount of lytic ontaminants in a clot test system, the problem was: where to begin when each of the components is suspect? An opening wedge seemed to be afforded hrough studies of the solution by SK alone of a hybrid clot made from human ibrinogen and bovine thrombin. The fibrinogen used was a 0.4 per cent olution in saline prepared from human plasma by the freeze-thaw method of Ware *et al.*[21] The source of bovine thrombin was the Upjohn commercial preparation, which was dissolved in saline with glycerol added as a stabilizer. SK caused rapid dissolution of this type of clot, showing that plasminogen was present as a contaminant. If the plasminogen were in the human ibrinogen, SK might have acted directly or upon a proactivator. If the plasminogen were in the bovine thrombin, a material must have been present n the human fibrinogen to produce the so-called universal activator. To ocate the source of plasminogen, the concentration of thrombin used to form he clot was varied. The results obtained are shown in TABLE 3. It will e seen that at every level of SK tested the lysis time lengthened as thrombin oncentration decreased. At 0.5 units of thrombin per assay tube and at optimal SK concentration the clots took 15 minutes to dissolve. This lysis ime represents only a small percentage of the activity observed with 4 units of thrombin, since lysis time is not a linear function of enzyme concentration. The inhibitory effect of an excess of SK is also shown by these data. This nhibition also occurred when highly purified SK was used.

Once we had established the fact that the activation of bovine plasminogen n the thrombin was a major source of error in this system, the purification f thrombin occupied our attention. It was finally found that thrombin ould be freed of plasminogen by the method of Milstone.[22] When the purified thrombin solution was added to human fibrinogen, 0.01 ml. formed solid clot within 20 sec. That this thrombin is free of lytic factors was hown by the lack of effect on the lysis time when the concentration of hrombin per assay was varied over a wide range. TABLE 4 shows the esults obtained at 2 levels of SK.

Table 3

Lysis Time of Clots Made with Human "Freeze-Thaw" Fibrinogen and Varying Concentrations of Bovine Thrombin in the Presence of Streptokinase

SK	Thrombin	
mg.	4 units	0.5 unit
0.008	7 min. 10 sec.	14 min. 50 sec.
0.016	7 min. 40 sec.	18 min. 25 sec.
0.024	7 min. 55 sec.	25 min. 10 sec.
0.032	9 min. 05 sec.	No lysis
0.040	9 min. 50 sec.	No lysis
0.080	10 min. 05 sec.	No lysis

Table 4

The Absence of Lytic Factors in Purified Thrombin

Human fibrinogen-buffer	SK		Bovine thrombin	Lysis time
ml.	ml.	units	ml.	
0.94	0.02	38	0.04	15 min. 20 sec.
0.96	0.02	38	0.02	18 min. 45 sec.
0.98	0.02	38	0.005	18 min. 0 sec.
0.86	0.10	330	0.04	28 min. 45 sec.
0 88	0.10	330	0.02	28 min. 45 sec.
0.89	0.10	330	0.01	28 min. 45 sec.

Turning back to our evaluation of the contamination present in the system it was shown that, in the presence of optimal amounts of SK, the clots mad from human fibrinogen and purified bovine thrombin dissolved in abou 20 min. In our assay procedure, the amount of enzyme necessary to dissolve a clot in 5 min. is determined. By successive dilution, it was foun that the 20-min. lysis time represents only 1 or 2 per cent of the activit necessary to dissolve the clot in 5 min. This is shown in figure 2. Thi clot was therefore considered to be sufficiently free of contamination for us in the assay of plasminogen. It might be asked how one could be certai that this system was virtually free of plasminogen in view of Astrup's clai that SK does not act directly upon plasminogen; if proactivator were missin the presence of plasminogen would not be demonstrable. It will be remembered, however, that the rapid dissolution of clots made with impure thrombin showed that the human fibrinogen used contained all of the factor needed for the production of the universal activator when SK was added.

The next set of experiments was carried out to determine whether th

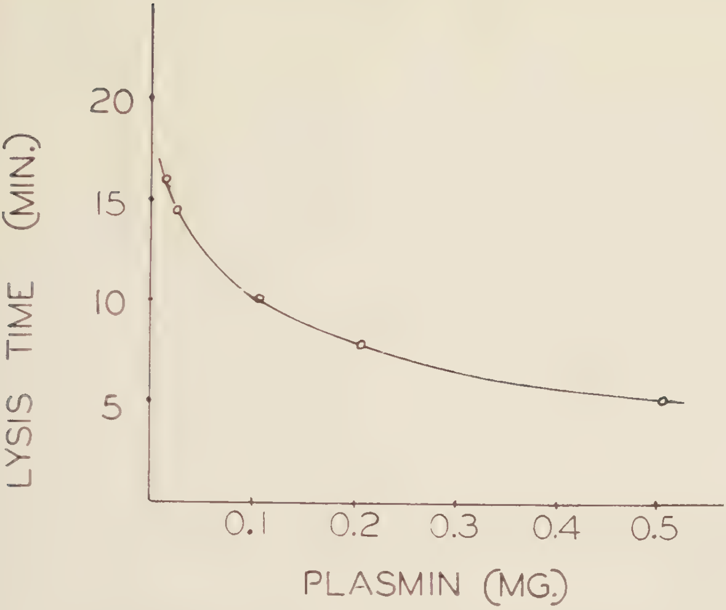

FIGURE 2. The solution by plasmin of a clot made from human fibrinogen and purified bovine thrombin. A lysis time of 20 min., the time taken by SK alone to dissolve the clot, represents less than 2 per cent of the activity measured in an assay based on a 5-min. lysis time.

small amount of fibrinolytic activity still demonstrable in our system was the result of an impurity in the SK. We were fortunate in being able to obtain a small amount of the new, highly purified SK preparation recently developed in the Lederle Laboratories. As may be seen in TABLE 5, the results with this material were similar to those achieved with Varidase, indicating that the SK used had not contributed appreciably to the contaminants of the system. These data also add weight to the evidence that the inhibitory effect of SK is a result of SK itself and not of a contaminant. Varying

TABLE 5
EFFECT OF THE CONCENTRATION OF PURIFIED STREPTOKINASE UPON LYSIS TIME

Fibrinogen-buffer	SK		Thrombin	Lysis time
ml.	ml.	units	ml.	
0.98	0.01	33	0.01	16 min.
0.97	0.02	66	0.01	17 min. 30 sec.
0.95	0.04	132	0.01	33 min.
0.93	0.06	198	0.01	No lysis
0.89	0.10	330	0.01	No lysis

the concentration of fibrinogen in the clot caused only minor changes in the lysis time, indicating that, despite the presence of the factor required to activate bovine plasminogen, under the conditions of our test system the fibrinogen contained negligible amounts of plasminogen.

Having established to our satisfaction that we had a clot-lysing test system that was essentially free of plasminogen, we now found it possible to obtain evidence as to the identity of the material that we had purified and called plasminogen.[5] The product obtained in each of the steps leading to the purification of plasminogen from Fraction III was tested, after the addition of SK, against the bovine clot that measures proactivator and against the human fibrinogen and purified bovine thrombin clot that measures plasminogen. For comparison, the amount of protein *N* necessary to dissolve the bovine clot in 5 min. and the purified clot in 7 min. was determined. These end points were selected arbitrarily for our own convenience; the results obtained are shown in TABLE 6. The ratios of the activities are listed in the last column of the table. No significant change was found in this ratio, about 6:1, at any stage of the purification procedure. This, we believe, is very strong evidence that the proactivator and plasminogen are identical substances. Similar results have been reported by Sherry.[16]

Similarly conducted tests showed that the material that we had isolated by alcohol fractionation and called plasmin[12] dissolved both impure and purified clots and was, therefore, a mixture of plasmin and activator. Tests carried out by F. B. Ablondi of the Lederle Laboratories (personal communication) using the lysine ethyl ester system yielded the same result. Upon the addition of alcohol to a mixture of human plasminogen and SK, the activator-to-plasmin ratio increased to 30:1 (TABLE 6), indicating that this procedure concentrated the activator. This result is not surprising since the alcohol fractionation procedure was developed using the impure clot and since conditions that were optimal for the purification of the activator were selected.

TABLE 6
RATIO OF PROACTIVATOR TO PLASMINOGEN DURING THE PURIFICATION OF PLASMINOGEN

	N to dissolve impure clot* mg.	*N* to dissolve purified clot† mg.	Ratio
Buffer extract	0.0210	0.1218	1:5.8
1st acid extract	0.0089	0.0516	1:5.8
2nd acid extract	0.0037	0.0148	1:4.0
Final product	0.0020	0.0114	1:5.7
Plasmin	0.0013	0.0413	1:32
Plasmin	0.0018	0.0506	1:28

* Bovine fibrinogen and unpurified bovine thrombin.
† Human fibrinogen and lytic-free bovine thrombin.

It has been suggested that the universal activator is a complex of which SK is a component.[16] This possibility was tested by labeling SK with I^{131}. The bound iodine did not alter the activity of the SK. After exhaustive dialysis to remove unbound iodine, the labeled SK was used to activate purified human plasminogen, and the plasmin and activator formed were isolated by alcohol fractionation. Since SK constitutes at most only about 25 per cent of the protein in Varidase, complete incorporation of the SK into the final product would be expected to give a maximum yield of 25 per cent of the starting radioactivity. As may be seen in TABLE 7, the results were definitely positive and suggest that an appreciable fraction of the SK was carried into the alcohol-precipitable fraction. As an added check, highly purified SK, which is believed to be about three to four times as pure as Varidase, was used in an additional test. The recovery of I^{131} was three times greater, corresponding to the increase anticipated. Although these experiments do not prove that the SK was associated with the activator rather than with plasmin, both of which are in the alcohol fraction, more recent experiments, about to be described, suggest that this is so.

Following the lead of both Sherry and Müllertz, who showed that plasmin is stable at *p*H 2, we were able to destroy the activator and to obtain a preparation that showed predominantly plasmin activity. Interestingly, these solutions showed a striking enhancement of plasmin activity, confirming the result obtained by Müllertz.[23] Plasmin has been precipitated from this solution and redissolved at *p*H 7.4. A few samples of this preparation have been tested in humans by Ambrus.[10] It has not yet been determined by isotopic experiment whether all of the SK is removed from the acid-treated plasmin, but preliminary experiments indicate a marked reduction in radioactivity of the final product (TABLE 7).

According to the data just presented, proactivator and plasminogen appear to be identical but, as I stated earlier, human fibrinogen contains proactivator in sufficient concentration to cause the lysis of a clot made with impure bovine thrombin, but shows only a trace of plasminogen activity. It was also found that, when SK is added to human plasminogen, both an activator and plasmin are formed and that these are chemically separable entities. How can these

TABLE 7
INCORPORATION OF I^{131}-LABELED STREPTOKINASE INTO ACTIVATOR

	Activating solution	Final solution	Per cent	Per cent of theoretical*
	Total c.p.m.	Total c.p.m.		
Varidase	1.47×10^6	1.43×10^5	10	40
Varidase	1.9×10^6	2.5×10^5	13	52
Purified SK	2.3×10^5	7.8×10^4	34	34
Purified SK	6.3×10^5	3.4×10^4†	5	5

* Assuming Varidase = 25 per cent SK and purified SK = 100 per cent SK.
† Acid-treated and reprecipitated plasmin.

apparently disparate data be reconciled and what theoretical fibrinolytic scheme will fit not only these observations, but also those made in other laboratories?

The seeming paradox that proactivator can be detected easily in human fibrinogen that is deficient in plasminogen and that these two enzymes are identical is readily resolved when it is recalled that the assay for proactivator is exceedingly sensitive and that minute amounts activate large quantities of plasminogen, whereas the assay of plasminogen requires relatively large amounts of this enzyme. By the elimination of thrombin and SK as possible sources of plasminogen, it was shown that traces of plasminogen still remain in the purified clot, causing the dissolution of these clots in 20 min. This small amount of plasminogen is sufficient to react with SK and produce the universal activator. Sherry has shown that only 0.3 gm. of enzyme nitrogen is needed for the demonstration of this activity. In our laboratory, we are currently producing plasmin that is virtually free of SK by the activation of plasminogen with very small amounts of activator.

A fibrinolytic scheme that is in accord with all of our observations is diagrammed in FIGURE 3. Proactivator and plasminogen are shown as a single substance. Plasminogen reacts with SK, probably stoichiometrically, to form a plasminogen-SK complex (possibly a plasmin-SK complex), the universal activator, which in turn catalyzes the conversion of plasminogen to plasmin. According to this theory, the complex contains SK in a form so altered that it will now activate the plasminogen of all species. The formu-

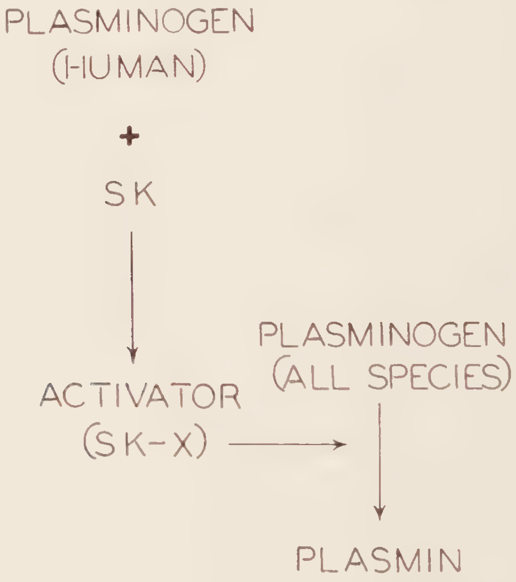

FIGURE 3. A fibrinolytic scheme showing the stoichiometric reaction of plasminogen and SK to form activator, which is an SK complex. The activator catalyzes the conversion of plasminogen to plasmin. Note that plasminogen plus SK produces two different enzymes.

lation presented is in complete accord with the scheme originally suggested by Sherry. We were impressed with his evidence in favor of this theory and did not feel that the work with synthetic substrates that led him to abandon it was sufficiently conclusive. The production of both activator and plasmin from one substance, and the different kinetics of activation of each are, we believe, understandable within the framework of the theory we propose. We have always felt that the similarity between the action of SK in the human and of staphylokinase in the dog[12] makes it unlikely that a unique factor from human blood is needed in the fibrinolytic system. It is our understanding that J. J. Hagan, F. B. Ablondi, and B. L. Hutchings (personal communication) have uncovered an artifact in the synthetic substrate experiments of Sherry, and that Sherry himself has recently proposed the original theory again; and we are gratified to be able to provide supporting evidence at this time.

Astrup and his associates maintain that proactivator is a discrete substance because of their findings of different ratios of proactivator to plasminogen in various fluids of the body. However, in view of the very different sensitivities of the assays involved and of the complex interactions between SK and plasminogen in which SK acts both as a necessary component and as an inhibitor, it seems to us at this time that the burden of proof is upon those who wish to introduce an as yet purely theoretical protein into the fibrinolytic system. Whatever the ultimate solution of this problem, I am pleased that we can suggest at this time a fibrinolytic scheme that is simpler rather than more complicated than its predecessors.

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Discussion of the Paper

Walter Troll (*New York University-Bellevue Medical Center, New York, N. Y.*): While I dislike to complicate matters, I cannot accept the attractive simplification that plasminogen is identical with proactivator. Synthetic substrates may not measure what you wish to measure, but they give decisive data that plasminogen preparations purified by your procedure contain at least two proenzymes. As we reported some time ago, heating such preparations at *p*H 9, withdrawal of samples at several time intervals, and activation with streptokinase reveal that arginine ester activity has fallen at a lower rate than lysine ester activity. Hence there must be at least two proenzymes in the purified plasminogen. We have also shown by more indirect means that activator activity is associated with lysine ethyl ester activity; lysine ethyl ester will inhibit activation of plasminogen to a greater extent than proteolysis, for example.[1]

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D. L. Kline: The question whether the different rates of activity against different amino esters prove that there are two substances in plasminogen is, in spite of what has been said, still open to interpretation. As I understand some of Ablondi's data, they show that *p*H is an exceedingly critical factor in amino acid ester assays. He has found that heat-inactivation studies show the presence of only one activity. This is one of the reasons why we went back to clot dissolution and tried to obtain a purified clot. This question is still open for discussion, of course, but my understanding at the moment is that there are data from heat-inactivation and kinetic studies to show that the activity against lysine and arginine ethyl esters is the same.

P. S. NORMAN (*Johns Hopkins University School of Medicine, Baltimore, Md.*): I should like to second most of the material that Kline has presented today. I have now used a caseinolytic assay for over two years, and I have prepared plasminogen according to Kline's method. Using this assay, I find that if one adds streptokinase to the purified plasminogen it then develops a strong proteolytic activity for casein. It also develops a strong activating activity for animal plasminogens. The ratios of activator and proteolytic activities of several different preparations made by this method are similar. I am able to remove the activating activity by the method described by S. Sherry, precipitation by 1 *M* salt solution at *p*H 2. Streptokinase can then be added to this material. This is an experiment that I think Sherry first described. The activating activity reappears. This point is still open to interpretation but, on the basis of results with a different method, I agree with Kline that the purification procedure gives both activator and plasminogen activities. I agree also that if you treat the product with acid to remove activator, as Kline has done, it seems most likely that you are removing streptokinase, because when you add streptokinase, you get your activator back. Furthermore, I find by my assay that increasing amounts of streptokinase inhibit the activity of plasmin. This is easily demonstrated.

S. SHERRY (*Jewish Hospital, St. Louis, Mo.*): Kline is to be congratulated on this group of experiments. They are certainly consistent with the observations that we published in 1954. I would like to add that subsequent work with Walter Troll indicated that there were probably two distinct factors, a proactivator and plasminogen.

In recent years, working with Miss N. Alkjaersig, we have gone back to the original view, that is, the one expressed by Kline, with which F. B. Ablondi and J. J. Hagan at the Lederle Laboratories also concur. They believe that there is not another substance, but that plasmin or perhaps plasminogen can actually react with streptokinase to form a complex, which is an activator for plasminogen. However, I believe that the observations of T. Astrup and S. Müllertz, and of Troll still deserve consideration. At the present moment I would keep an open mind as to whether there are two different substances causing this two-step reaction or whether there is one substance involved in a two-step activation procedure.

F. HAUROWITZ (*Department of Chemistry, Indiana University, Bloomington, Ind.*): Is there any connection between plasminogen and one of the fractions of complement?

D. L. KLINE: I have had no personal experience with that problem.

E. L. BECKER (*Division of Immunology, Walter Reed Army Institute of Research, Washington, D. C.*): I think I can answer that question quite definitely. The complement and the plasminogen are completely different. For example, I have been able to remove from the complement fraction by adsorption on fibrin practically all of the plasminogen activity and yet retain the greater part of the first component. There is other evidence along this line. The activity of the first component is not changed on the addition of streptokinase if one has the proper conditions.

ACTIVATION OF PLASMINOGEN*

By Sten Müllertz

The Biological Institute, Carlsberg Foundation, Copenhagen, Denmark

Plasmin is the proteolytic and fibrinolytic enzyme of blood. It is normally present in blood as an inactive precursor, plasminogen. Activation of plasminogen to plasmin may occur in the animal organism under various conditions. After death strong fibrinolytic activity is often found in human blood. *In vitro*, a conversion of plasminogen into plasmin can be effected by dilution or precipitation procedures or by treating serum, plasma, or globulin fractions with chloroform and related substances, with tissue or tissue extracts, or with trypsin. Culture filtrates from certain streptococci and staphylococci contain streptokinase and staphylokinase, substances that effect the activation of plasminogen. Before 1952, among several discrepancies noted in the behavior of these enzymes, the most obvious were: the difference in reaction of animal and human plasminogen to the activators, streptokinase and tissue; and the varying reaction of fibrin substrates and other protein substrates to the active substances. Animal plasminogens were partially or totally resistant to streptokinase, while human plasminogen was activated quickly and completely. On the other hand, tissue activator produced activities of the same magnitude in all plasminogens, whatever substrates were used to determine the activity. When a bovine fibrin substrate was used, the lytic effect of human globulin activated by streptokinase was far greater than that of human or animal globulin activated by tissue activator. Any hypothesis of the mechanism of activation of plasminogen should supply a reasonable explanation of these discrepancies. Surveys of earlier and recent literature on these subjects are given by Macfarlane and Biggs (1948), Astrup (1956), and Müllertz (1956).

During recent years, information concerning the mechanism of activation of plasminogen has been obtained primarily from studies on the effect of streptokinase, on the activator in spontaneously fibrinolytic human blood, and on the influence of fibrin and inhibitors on the activation process in blood.

Studies on the Effect of Streptokinase

Independent observations of Geiger (1952), Müllertz (1952), and Müllertz and Lassen (1953) have shown that a factor in addition to plasminogen is needed for the formation of plasmin by streptokinase. Geiger (1952) prepared mixtures containing (1) streptokinase; (2) the euglobulin fraction from human plasma heated at 56° C. for one hour; and (3) the euglobulin fraction from fresh serum of guinea pig, rabbit, chicken, or duck. In all instances the proteolytic activity of the mixtures exceeded the sum of the amounts that were produced on the addition of streptokinase to the separate globulin fractions. The experiments clearly showed that more than one component is needed for the formation of plasmin.

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Müllertz and Lassen (1953) studied especially the effect of streptokinase on human and bovine globulin. We found that human streptokinase-activated globulin produced a strong lytic effect in a bovine plasminogen-containing fibrin substrate, but a relatively weak effect in a plasminogen-free fibrin substrate. A very low proteolytic activity was produced by addition of streptokinase to human globulin or to bovine globulin, while a strong activity was obtained in a mixture containing streptokinase, minute amounts of human globulin, and bovine globulin. Bovine globulin was shown to contain large amounts of plasminogen that could not be activated completely by streptokinase. A complete conversion was produced by the further addition of small amounts of human globulin. The experiments showed that streptokinase alone converted only a small part of the bovine plasminogen into plasmin, but transformed a larger part in less purified than in purified preparations. A factor necessary for the formation of bovine plasmin by streptokinase was probably present in insufficient amount in bovine material and lost during purification. The activation of bovine plasminogen by human globulin and streptokinase was explained by the formation of large amounts of an activator of plasminogen in human globulin by streptokinase. The following reactions were suggested, but their type was not specified:

$$\begin{tikzcd} & & \text{plasminogen} \arrow[dd] \\ \text{proactivator} \arrow[r, "\text{streptokinase}"] & \text{activator} \arrow[r] & {} \\ & & \text{plasmin} \end{tikzcd}$$

According to the hypothesis, bovine serum and especially preparations of bovine plasminogen contain small amounts of proactivator and relatively large amounts of plasminogen. Human serum and globulin contain large amounts of proactivator and relatively small amounts of plasminogen. The experimental results were confirmed by Sherry (1954), who found that streptokinase produced a weak proteolytic activity in bovine globulin and in dog and rabbit plasma, and that the addition of small amounts of human globulin resulted in the development of a strong proteolytic activity.

Further evidence supporting the hypothesis of a proactivator-activator system in human blood is available. Attempts have been made to prepare human plasminogen free from proactivator. Although a complete separation has not been achieved, varying proportions between proactivator and plasminogen have been observed. The presence of two different esterase precursors in human globulin was suggested by Troll, Sherry, and Wachman (1954) and established by Troll and Sherry (1955). In the latter study, one component was found to attack lysine ethyl ester (LEe) and another component to attack tosyl arginine methyl ester (TAMe). Studies on the inhibition of streptokinase activation of human globulin related the LEe esterase activity to the plasminogen activator and identified the TAMe esterase activity with plasmin. I was able to obtain (Müllertz, 1955) a complete activation of human plasminogen at concentrations of streptokinas about fifty times smaller than those used for a maximal formation of activator. In the same way activation of human globulin by varying concentrations of streptokinase revealed that the LEe and TAMe esterase activitie bore different relations to the streptokinase concentration (Troll and Sherry 1955). Studies by these workers on heat denaturation of human globuli revealed different stabilities for the precursors of the LEe and TAMe esterase

Studies on the Activator in Spontaneously Fibrinolytic Blood

Schmitz (1936) originally suggested the presence in blood of a kinase fo plasminogen. Lewis and Ferguson (1951) prepared from normal dog bloo two fractions that remained inactive when stored separately, while stron fibrinolytic activity developed after incubation of a mixture of the tw fractions for several weeks at 5° C. I studied (Müllertz, 1953b) the stron fibrinolytic effect produced in a bovine fibrin substrate by globulin solution prepared from spontaneously lytic blood obtained from human subjects afte death from anoxia. While strong fibrinolytic effects were produced in normal plasminogen-containing bovine fibrin substrate, only weak effect were produced when the plasminogen of the substrate had been destroye by heating. By adding bovine plasminogen to a solution of active huma globulin, forty times more plasmin than originally present was formed. similar but lower increase in plasmin activity was obtained with activ globulin obtained from human patients subjected to electrically induced convulsions in psychiatric therapy. These results indicate the presence of a activator of plasminogen in the active samples. In further studies (Müllert 1954b) the properties of different activator preparations were studied mor closely. Three different activator preparations were compared: (1) a preparation made by adding an excess of streptokinase to globulin from norma human blood (FIGURE 1); (2) a preparation made by adding minimal amount of streptokinase to globulin from normal human blood and showing a simila plasminogen-activating potency to (3) a preparation of globulin from huma fibrinolytic blood obtained post mortem (FIGURE 2). The activator presen in preparation No. 1 (FIGURE 1) was completely destroyed at acid reaction while the activators present in the other two preparations showed a comparatively high stability at acid reaction and characteristic and almos identical *p*H stability curves (FIGURE 2). The similar stability of the activator in preparations No. 2 and No. 3 indicated the presence in spontaneousl active blood of a factor with the same effect as streptokinase. The presenc of this factor in a number of such preparations was demonstrated by th strong increase in activator activity produced by addition of normal globuli (that is, proactivator) to different globulin fractions from active blood. I a large series of subsequent experiments, however, only slight increases i activator activity could be obtained with some preparations after additio of proactivator, showing that only small amounts of the streptokinase-lik component and large amounts of proactivator were present. Astrup an Sterndorff (1956) also report the presence in certain tissue extracts of sma amounts of a factor that effects the conversion of proactivator into activato

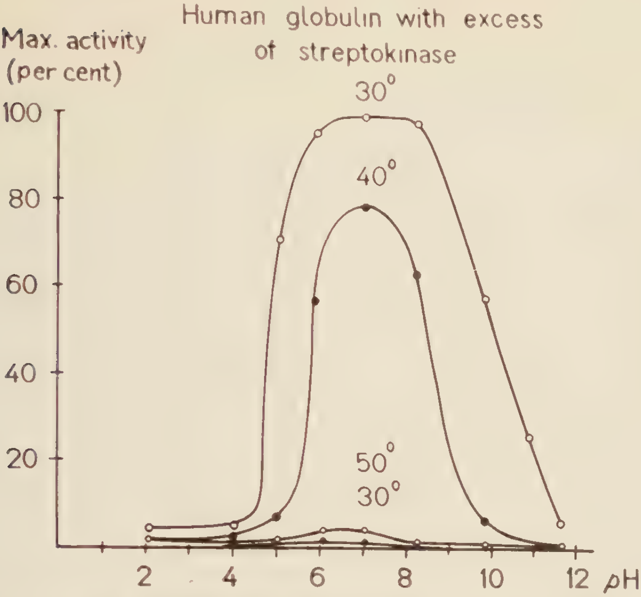

FIGURE 1. Residual plasminogen-activating potency after heating the preparation for 30 min. at different temperatures and *p*H. Standard fibrin plate method.

This factor is different from tissue activator, which attacks plasminogen directly.

These observations suggest that the mechanism of formation of activator in spontaneously fibrinolytic blood is similar to that described for streptokinase.

Methods for Estimating Activator and Proactivator

Astrup and Permin (1947) were the first to demonstrate that purified bovine fibrinogen contains large quantities of plasminogen. They showed that the lysis produced in a substrate of clotted bovine fibrinogen by tissue was caused by activation of the plasminogen of the substrate. These observations have been confirmed repeatedly by means of the tissue activator (Lewis and Ferguson, 1950; Lassen, 1952) and by activators in human blood (Müllertz, 1953b; Müllertz and Lassen, 1953; Sherry, 1954). It can be concluded that the lysis of clotted bovine fibrinogen is produced either directly by proteolytic (fibrinolytic) enzymes that split the fibrin molecule, or indirectly by activators of plasminogen that convert plasminogen in the substrate into plasmin, with a subsequent splitting of fibrin. Both plasmin and activators can be estimated by means of this substrate.

A detailed description of the fibrin plate method and its use has been presented by Astrup and Müllertz (1952). In this method the active solutions are placed on the surface of a thin layer of clotted bovine fibrinogen, and the activity is measured by the area of the lysed zones. A quantitative estimation of the fibrinolytic activity of a diluted solution of streptokinase-

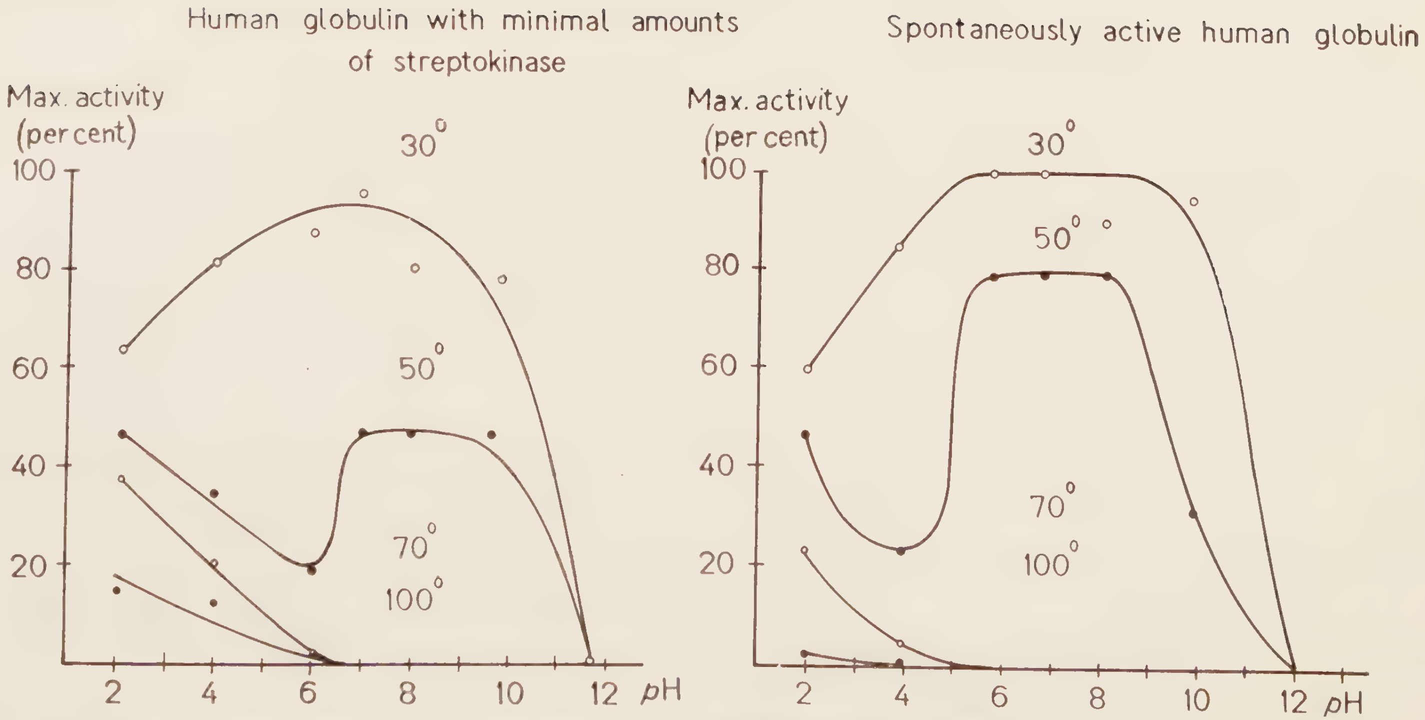

FIGURE 2. Residual plasminogen activating potency after heating preparations for 30 min. at different temperatures and *p*H. Standard fibrin plate method.

activated human globulin (that is, activator) was obtained by means of dilution curves and interpolation on a reference curve. Further studies (Müllertz, 1955) showed that the plasmin present in the highly diluted solutions of activator used in the method produced no significant lysis in the plasminogen-free bovine fibrin substrate prepared by heat denaturation of the fibrin, as described by Lassen (1952). Sherry (1954) confirmed the observation that the fibrinolytic assay for streptokinase-activated human plasmin, as generally employed using determinations of lysis time in bovine fibrinogen clotted by thrombin, measures the activation of plasminogen in the substrate rather than the fibrinolytic activity of the human plasmin of the sample. It may be concluded that previous studies on the interaction of streptokinase, human plasminogen, and plasmin, performed by means of a bovine fibrin substrate, are pertinent primarily to the proactivator-activator system and only to a very slight degree to plasminogen and plasmin. The lysis time method is less satisfactory, since controls cannot be obtained to show the lytic effect of plasmin in the samples. Such controls can be obtained in the fibrin plate method.

In our laboratory there has been developed (Müllertz, 1955) an empirical method for the assay of the activator that involves appropriate controls and requires short reaction periods. In this method activator is added to a solution containing bovine purified plasminogen and casein in excess. During incubation for 20 minutes at 37° C., two reactions proceed simultaneously: the activation of plasminogen and the splitting of casein by the plasmin formed. The increase in optical density at 275 mμ of acid-deproteinized samples removed from the mixture is determined and is converted into activator concentration by means of a reference curve. Proactivator may be measured as activator after addition of an excess of streptokinase (Müllertz, 1954a).

Formation of the Activator

Early studies by Christensen and MacLeod (1945) and by Remmert and Cohen (1949) had revealed a catalytic effect of streptokinase. Wasserman (1952), on the other hand, found that streptokinase combined stoichiometrically with human plasminogen. However, the activity was determined by measurements of lysis time on clotted bovine fibrinogen, and his results with this substrate probably are pertinent primarily to the formation of activator by streptokinase and proactivator. His experiments strongly support the assumption that these components react stoichiometrically.

The destruction of the plasminogen activating potency of streptokinase-activated human globulin at acid reaction, and the restoration of activator activity by additional streptokinase led Troll and Sherry (1955) to propose a reversible stoichiometric reaction between streptokinase and proactivator. Their observations strongly support this hypothesis, even if our present knowledge does not allow us to account quantitatively for the results of the experiments.

The reaction between streptokinase and proactivator was studied (Müllertz, 1955) by means of a method for quantitative assay of the activator.

At suboptimal concentrations of streptokinase only a partial transformatio of proactivator into activator was obtained. In further experiments the amount of activator formed by using different proportions of streptokinase and proactivator was determined. By varying the concentration of streptokinase in the presence of a fixed concentration of proactivator, the equivalent concentrations were found. In two experiments the concentrations of streptokinase and of proactivator, respectively, were varied above and below the equivalent concentration in the presence of a fixed concentration of the other component. The results indicated a reversible reaction proceeding to an equilibrium. The effect of dilution on the equilibrium was studied (1) by adding streptokinase to proactivator in equivalent concentrations and allowing the reaction to proceed to equilibrium in concentrated and in dilute solutions, and (2) by diluting a concentrated solution containing the components in equilibrium. In the first case a slightly smaller amount of activator was formed in the dilute solution, while in the second experiment no difference was observed. A calculation of the theoretical effect of dilution showed that the following reaction was the most probable:

$$\text{streptokinase} + \text{proactivator} \rightleftharpoons \text{activator} + \text{inactive component}$$

Other observations (Müllertz 1954a) may suggest an even more complicated reaction. The proactivator in normal human globulin and in active globulin prepared from post-mortem blood showed a markedly different stability at different *p*H and temperatures (FIGURE 3). Normal human globulin was found to yield an unchanged amount of activator on the addition of streptokinase after incubation for 20 hours at 22° C. and *p*H 7.4 while active globulin yielded rapidly decreasing amounts of activator (after addition of streptokinase) during incubation. The results indicated that a component that was involved in the formation of activator by streptokinase was consumed during the incubation. As the activator did not simultaneously increase, this component was probably not identical with the proactivator. Furthermore, the amount of activator formed in a mixture o streptokinase, spontaneously active globulin, and normal globulin was considerably larger than the sum of the amounts of activator formed by streptokinase in separate samples of the globulin preparations (TABLE 1). This observation suggested that at least one of the samples contained proactivato that was not converted completely into activator by addition of an excess of streptokinase. As a tentative explanation it was proposed that a reaction between streptokinase and another component in human globulin precedes the reaction with the proactivator leading to the formation of activator.

Formation of Plasmin by the Activator

Previous studies have led to different concepts of the mechanism of formation of human plasmin by streptokinase. Christensen (1945) and Christensen and MacLeod (1945) used gelatin, and Ratnoff (1948) and Remmert and Cohen (1949) used casein as substrates for the assay of plasmin. These studies indicated convincingly that the conversion of plasminogen was

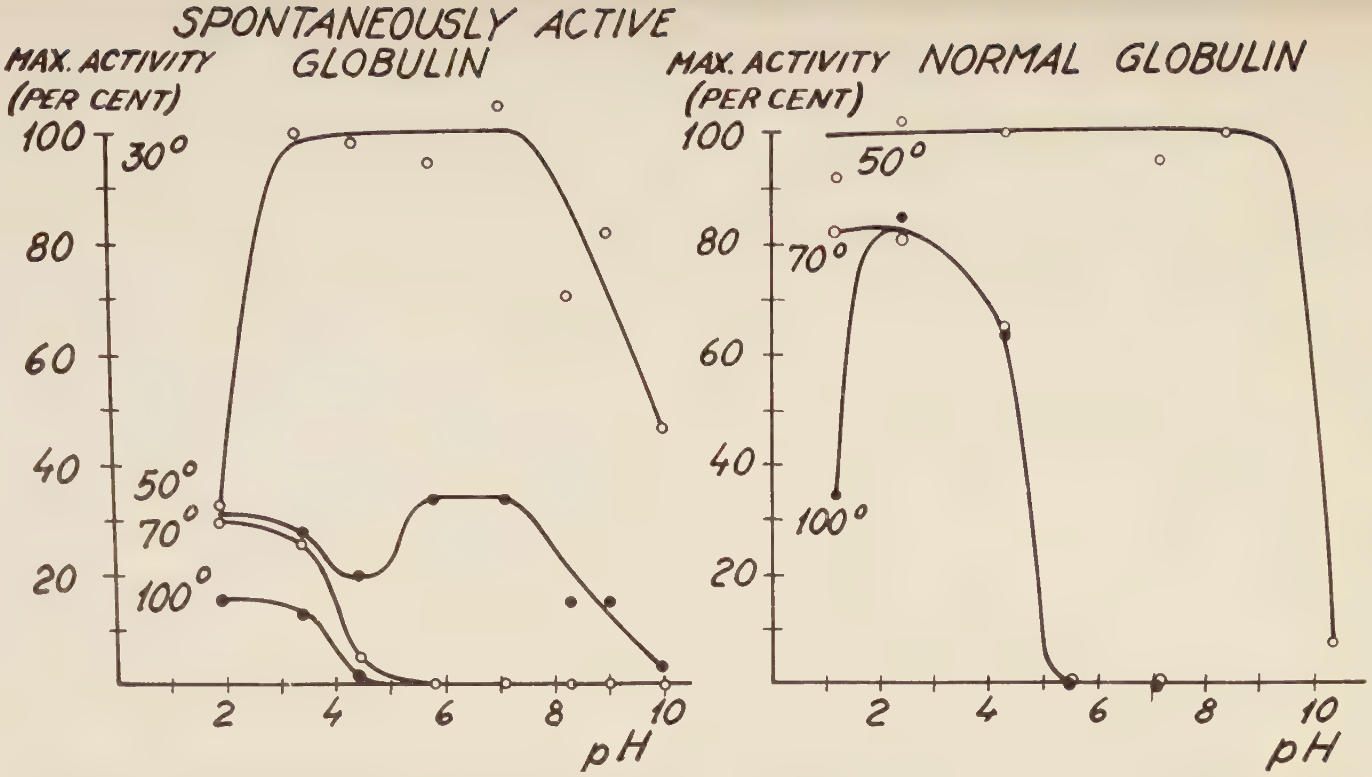

FIGURE 3. Residual concentration of the precursor components of the plasminogen activator in spontaneously fibrinolytic and in normal inactive globulin. Determined as plasminogen activator after addition of an excess of streptokinase. Standard fibrin plate method.

Table 1
Activator Activity of Mixtures of Active Globulin, Normal Globulin and Streptokinase
Standard Fibrin Plate Method

Mixtures	Activator activity (%)	
	Sample A	Sample B
Active globulin, freshly prepared Streptokinase	100	100
Active globulin, incubated Streptokinase	26	65
Normal globulin Streptokinase	20	13
Active globulin, freshly prepared Normal globulin Streptokinase	160	145
Active globulin, incubated Normal globulin Streptokinase	125	130

catalyzed by streptokinase. Wasserman (1952) used a bovine fibrin substrate, and his results are pertinent primarily to the interaction of streptokinase and proactivator. We (Müllertz and Lassen, 1953) showed that at least two reactions were involved in the formation of plasmin by streptokinase and concluded that, in view of earlier observations, at least one of the reactions must be a catalytic reaction. Our preliminary results indicated a stoichiometric reaction between activator and bovine plasminogen, but this conclusion is probably not valid because of the lability of the reacting components under the conditions established. Troll and Sherry (1955) confirmed the first-order kinetics for the formation of human plasmin by streptokinase. They stopped the reaction by a precipitation technique before the assay of plasmin by tosyl arginine methyl ester and by casein. From this observation they suggested that activator catalyzed the transformation of plasminogen into plasmin. Since only human material was used, the effect of activator on a proactivator-free plasminogen could not be investigated. Some information on the mode of action of activator on bovine plasminogen (free from proactivator) was presented by Müllertz (1955). The time relationship of the formation of bovine plasmin at different activator concentrations at 22° C. and at 5° C. was followed. In all instances a progressive activation of plasminogen at small activator concentrations and a slow deterioration of plasmin resulted in approximately identical plasmin activities. These results suggest a catalytic reaction. Recent experiments by

Lassen (1956) have strongly supported the enzymatic nature of the effect of activator on bovine plasminogen.

Influence of Fibrin and of Inhibitors on the Activation of Plasminogen in Blood

Controversial results have been reported in studies on the splitting of fibrin and fibrinogen by different active preparations from blood. In a few experiments the effect of plasmin on purified preparations of fibrinogen and fibrin has been studied. Permin (1949, 1950) used a bovine plasmin activated by tissue. Ratnoff (1953) used a bovine, chloroform-activated plasmin and a human plasmin prepared by successive treatments of globulin with streptokinase and chloroform. The bovine preparations—and possibly the human sample contain no activator, or only small amounts of it. They produced almost the same rate of fibrinogenolysis as fibrinolysis in bovine and human purified preparations. Therefore it is highly probable that plasmin splits pure fibrinogen and fibrin at equal rates.

In most studies, streptokinase or preparations of activator have been added to plasminogen-containing solutions of fibrinogen or fibrin (that is, clotted fibrinogen). Under these conditions the development and rate of fibrinogenolysis and fibrinolysis depend on the rate of activation of plasminogen as well as on the rate of the subsequent splitting of the substrate. In a number of these experiments fibrin and fibrinogen were lysed in almost the same length of time, indicating that both the rate of activation and the rate of the splitting of the substrate were comparable in both instances. In other experiments, however, fibrin was split in a shorter length of time than was fibrinogen.

More uniform results have been observed when the splitting of fibrinogen and fibrin has been studied in plasma instead of in partially purified preparations. Thus, human streptokinase-activated globulin and plasma were found to induce fibrinolysis, but no fibrinogenolysis, in human plasma (Fantl and Fitzpatrick, 1950; Ratnoff, 1953,; and the same results were observed with spontaneously fibrinolytic human globulin obtained *in vivo* (Ratnoff, 1953; Bidwell, 1953).

The fact that fibrinogenolysis was absent or very slow in the presence of plasma or serum, while under certain conditions it had been found to proceed at about the same rate as fibrinolysis in purified preparations, led me to suggest (Müllertz, 1953a) that inhibitory substances might be responsible for the observed discrepancies. I made a qualitative estimation of the inhibitor content of different samples of post-mortem plasma, and found the rates of fibrinolysis and fibrinogenolysis to be almost identical in samples containing little or no inhibitor, while fibrinolysis, but no fibrinogenolysis, was observed in samples with a high inhibitor content. It may be pointed out that the different rates of fibrinogenolysis and fibrinolysis may be explained by an influence of inhibitors on the activation of plasminogen as well as on the action of plasmin. However, a number of studies have indicated that the influence of fibrin and of an inhibitor on the activation process is the primary cause of the observed discrepancies.

Fibrin seems to be of special importance for the activation of plasminogen The activator present in post-mortem plasma was found to be strongly adsorbed to fibrin and released again after lysis of the clot (Müllertz, 1953a) The activator in active globulin from living human subjects (Müllertz, 1953b) and in streptokinase-activated human globulin (Müllertz, 1953a) was also found to be strongly adsorbed to fibrin. Ratnoff (1953) concluded that plasmin digested fibrin and fibrinogen at equal rates, while fibrin was destroyed much faster than fibrinogen in spontaneously fibrinolytic human blood and on the addition of streptokinase to human plasma. He suggested that the activation of plasminogen was accelerated by the process of coagulation possibly through an effect of the fibrin surface.

The presence of an inhibitor for the activation of plasminogen has been suggested by Schmitz (1937) and by Lewis and Ferguson (1951). The presence of an antiactivator in blood was demonstrated in preliminary experiments (Müllertz, 1954b); a crude sample of bovine globulin inhibited the activation of bovine plasminogen by the activator in human streptokinase-activated globulin, and further experiments showed that the inhibitory effect of bovine globulin on the activation process was markedly reduced in the presence of fibrin.

Bidwell (1953) followed the progressive lysis of fibrin clots in diluted samples of active human plasma obtained *in vivo*. She found that the amount of fibrin remained constant for some hours, and then began to diminish rapidly, and that the lysis was complete in a relatively short time All these observations indicate that plasmin is not originally present in spontaneously lytic blood and that plasmin is not formed by addition of activator to plasma or to preparations with a high inhibitor content. A similar view is supported by Bastian and Hill (1956) in a recent preliminary report.

It may be assumed that the activator is bound by antiactivator in fluid blood. Consequently no plasmin is formed and no splitting of fibrinogen takes place. In the presence of fibrin, activator is adsorbed to the fibrin surface, whereby the effect of the antiactivator is probably reduced; and the activation of plasminogen occurs, with subsequent fibrinolysis.

Discussion

The resistance of plasminogen in plasma and in globulin fractions from animal blood to streptokinase has received different explanations. Thus, it has been ascribed to: (1) absence of plasminogen; (2) high levels of antistreptokinase, a specific immune antibody developed during streptococca infections; (3) high levels of an antiprotease that has been found to occur in rather high concentrations in animal sera; or (4) a strict species specificity of streptokinase. However, none of these explanations seems satisfactory The activity against plasminogen-free protein substrates produced by streptokinase in human globulin was comparable to that occurring spontaneously or after staphylokinase activation in globulin fractions from different animals and to that occurring either spontaneously or after treatment with tissue activator or chloroform in globulin fractions from ox an other animals. The concentration of a specific antistreptokinase should be insignificant in the presence of an excess of purified kinase and does not account for the effect of further addition of slight amounts of human thrombin or globulin. A high content of antiprotease may also be omitted from consideration, as this inhibitor should be as active against plasmin produced by other means as by streptokinase. A strict species specificity of streptokinase also seems improbable, as small proteolytic and fibrinolytic activities have been produced by purified streptokinase in fractions from animal blood.

The resistance is most easily explained by a low content of proactivator and perhaps also high concentrations of inhibitors, especially antiactivator, in animal blood.

The activation of animal plasminogen by mixtures of streptokinase and human globulin may also be explained through the proactivator-activator system. Other explanations have been proposed, however. An autocatalytic activation of plasminogen or a catalytic effect of human plasmin on animal plasminogen is contradicted by various observations. Thus, plasmin and plasminogen from ox blood (Astrup, 1951) and from dog blood (Lewis and Ferguson, 1952) could be kept in the same solution without any evidence of interaction. In both instances the plasminogen could be activated by other means. Human plasmin that had been heated at acid reaction to remove any activators of plasminogen showed no effect on human or bovine plasminogen, although a strong activity could be produced by a plasminogen activator in the presence as well as in the absence of plasmin (Müllertz, 1955). Sherry (1954) claimed that the activation of animal plasminogen was caused by a complex of streptokinase and human plasmin or, alternatively, that it was due to a specific binding of a hypothetical inhibitor in animal sera by human plasmin, whereby animal plasminogen was released for activation by streptokinase.

These theories do not explain why streptokinase activates human plasminogen while animal plasminogen is more or less resistant, the degree of susceptibility to activation depending on the purity of the preparation. Furthermore, they supply no explanation of the activating effect of spontaneously fibrinolytic human globulin on animal plasminogen. In particular, the observations on the effect of streptokinase on separate and mixed samples of spontaneously fibrinolytic and normal human globulin appear unexplainable unless one postulates that other factors in addition to plasminogen are necessary for the formation of plasmin by streptokinase. In a subsequent paper Troll and Sherry (1955) adopted the hypothesis of a proactivator-activator system.

Various observations published recently suggest a change in concept concerning the mechanism of fibrinolysis under physiological conditions in blood. The concept presented here may serve as a working hypothesis for further studies.

Some studies suggest that the development of fibrinolytic activity in blood is associated with the state of the vascular system. It is probable that any change that leads to an increased permeability of the vascular endothelium and release of tissue components at the same time entails an increase in the potential clotting tendency and in the potential fibrinolytic activit of blood. A factor from tissue apparently combines with components i blood to form plasminogen activator. Blood from normal human subject has been found to contain a potential fibrinolytic activity that is increase under certain conditions. It is probable that some activator is continuousl formed by small amounts of the tissue component released into blood, s that small amounts of activator are present in blood under normal conditions. In fluid blood all active substances circulate in a neutralized state Consequently there is no activation of plasminogen and no splitting o fibrinogen or of other plasma proteins. Activator is bound by a specifi inhibitor, antiactivator. Activator is strongly adsorbed on fibrin from th fluid circulating blood. In this way activator accumulates on the surfac of fibrin deposits, and local high concentrations can be reached, althoug the concentration of activator in the blood is very low. The effect of inhibitors against activation and perhaps also against the effect of plasmin i reduced by the adsorption of activator and plasmin to the fibrin substrate The activator-antiactivator compound may dissociate when activator i adsorbed on fibrin. The activation of plasminogen takes place on the fibri surface, and the fibrin is subsequently digested by the plasmin formed in th clot. By the lysis of fibrin, plasmin and activator are released into bloo and probably neutralized there by their respective inhibitors. Simultaneously some deterioration of the rather labile components takes place.

Our present knowledge of the proteolytic enzyme system in blood is fragmentary. Further studies are needed to establish the properties and th mechanism of interaction of the components already known to exist. Futur investigations may possibly reveal other components and other mechanism involved in the sequence of events that ultimately produce the lysis of th fibrin clot.

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BIOCHEMICAL, EXPERIMENTAL, AND CLINICAL STUDIES O PROTEOLYTIC ENZYMES: WITH PARTICULAR REFERENCE TO THE FIBRINOLYTIC ENZYME OF HUMAN PLASMA*

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Following the successful introduction of intrathoracic injections of streptokinase for the dissolution of clotted hemothorax[1] considerable interest ha been aroused in the development of fibrinolytic enzymes for the therapy o thromboembolic disease in man. Two approaches have received seriou consideration: (1) the use of highly purified proteolytic enzymes capable o dissolving fibrin *in vitro* at neutral *p*H;[2, 3, 4] and (2) the activation of th naturally occurring fibrinolytic and proteolytic enzyme of mammalia plasma.[4, 5] To date, our studies have been primarily concerned with a investigation of the fibrinolytic enzyme of mammalian plasma. As a background for the *in vivo* studies in man, we have also investigated some of th fundamental aspects of the plasma fibrinolytic enzyme system of mammalia plasma. It is the purpose of this report to review some of these studies, a well as to describe some of the effects of intravenously infused streptokinas in patients.

As a result of the studies of Christensen,[6] and of Kaplan,[7] it is now wel known that human plasma contains a precursor of a proteolytic enzym termed plasminogen or profibrinolysin. In the presence of a kinase or spontaneously, when inhibitors are removed, this serum globulin is converted int the active enzyme—plasmin or fibrinolysin.

Although it digests fibrin into several soluble incoagulable fragments† plasmin is not restricted in its action to fibrin. It has been shown to ac also upon fibrinogen, casein, gelatin, β-lactoglobulin, azocoll hide powder,[1] Ac-globulin,[1, 12] at least two components of serum complement[13] and, perhaps prothrombin.[14]

Methods for Assaying Plasmin

Plasmin is commonly assayed at present by any one of three methods fibrinolysis,[15] casein proteolysis,[16] or the hydrolysis of synthetic substrates.[1] In FIGURE 1 is shown the rate of lysis of a standard bovine fibrinogen thrombin clot as a function of plasmin concentration. A direct relationshi exists between the reciprocal of the lysis time and the enzyme concentration

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* Supported by grants from the National Heart Institute, Public Health Servic Bethesda, Md., and Lederle Laboratories Division, American Cyanamid Co., Pearl Rive N. Y.

† Although the original studies of Holmberg[8] suggested that the action of plasmin o fibrinogen is limited to a single split into 2 large fragments, our studies utilizing 2-phas paper chromatography, as originally developed by Franklin *et al.*,[9] indicate a more extensiv digestion of these substrates. Shulman, utilizing ultracentrifugal and electrophoreti techniques, has made similar observations.[10]

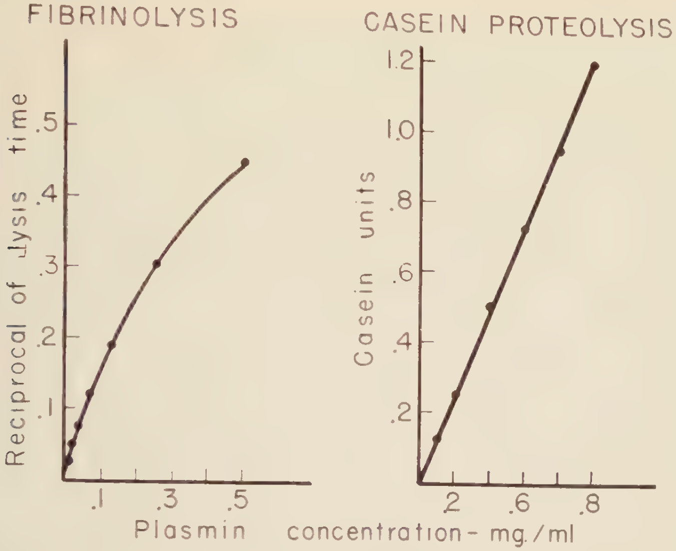

FIGURE 1. Fibrinolysis and casein proteolysis as a function of plasmin concentration. The plasmin was prepared by spontaneous activation of plasminogen in 50 per cent glycerol.

At very high concentrations of plasmin, the digestion of fibrinogen is so rapid that the addition of thrombin does not result in the formation of a clot. FIGURE 1 also demonstrates the linear relationship observed when casein proteolysis, as measured by release of acid-soluble tyrosine, is used as a measure of plasmin activity. Assays other than fibrinolysis are particularly important when plasmin is assayed in the presence of activators, inasmuch as almost all animal fibrinogen preparations used in fibrinolysis studies are contaminated with significant amounts of plasminogen.[18, 19]

In FIGURE 2 are shown the characteristics of the action of plasmin on synthetic lysine and arginine esters as originally described by Troll, Sherry, and Wachman.[17] The synthetic lysine ester employed was the unsubstituted lysine ethyl ester referred to as LEe, and the synthetic arginine ester was tosyl arginine methyl ester referred to as TAMe. Benzoyl arginine methyl ester (BAMe) is also an excellent substrate for plasmin, and we have utilized this substrate in our more recent studies.

Physicochemical Characteristics of Human Plasmin and Plasminogen

Reliable studies on some of the characteristics of human plasminogen and plasmin have been hampered by the lack of techniques for purification of these substances. Kline,[20] by modifying Christensen's method of acid extraction of Fraction III,[21] has now made available a simple procedure for obtaining preparations of human plasminogen of high specific activity. By allowing these preparations to activate spontaneously to completion in the presence of stabilizing agents under conditions to be described below, prepa-

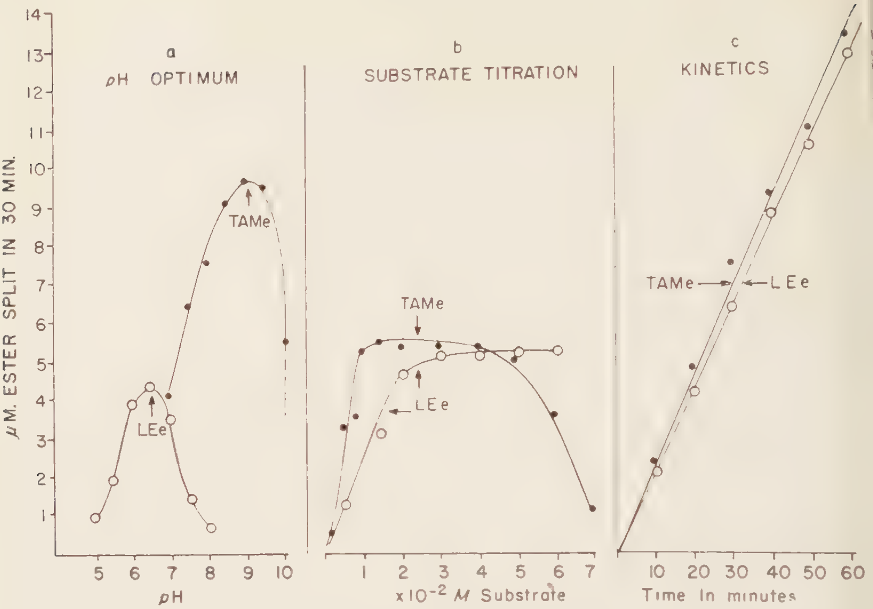

FIGURE 2. Enzyme characteristics. (a) pH optimum curves with a plasminogen preparation obtained by isoelectric precipitation of Fraction III[6] and containing 62.4γ o nitrogen per ml. of digestion mixture activated with 1000 units of streptokinase. The substrates, tosyl arginine methyl ester (TAMe) and lysine ethyl ester (LEe), were 0.02 M 0.25 M *tris*-(hydroxymethyl)aminomethane (TRIS) buffers were used for the former an 0.1 M imidazole buffers for the latter. (b) Substrate titration curves. A human globuli preparation, prepared according to Milstone,[8] containing 126γ of nitrogen per ml. o digestion mixture, and activated by 1000 units of streptokinase, was used as the enzym source. TAMe was incubated at pH 9, 0.25 M TRIS buffer, and LEe at pH 6.5, 0.1 M imidazole buffer. (c) Kinetics of ester digestion of 0.02 M TAMe at pH 9, 0.25 M TRIS buffer, and of 0.04 M LEe at pH 6.5, 0.1 M imidazole buffer with 0.1 ml. of plasma (889γ o nitrogen) activated with 1000 units of streptokinase as the source of enzyme All experiments were carried out at 37° C. Esterase activity was determined by the metho described in the text. (Reprinted from W. Troll, S. Sherry, and J. Wachman; *Journal o Biological Chemistry*, 1954, vol. 208, p. 85.)

rations of human plasmin of high purity can be obtained. Utilizing these newer techniques, a number of preparations of human plasminogen an spontaneously activated plasmin were prepared for physicochemical stud by S. Shulman. The preliminary observations made on these preparation are shown in TABLE 1.

On ultracentrifugal analysis, the plasminogen and plasmin preparation appear to be quite homogeneous. Plasminogen has a sedimentation constan of 4.28 and plasmin a sedimentation constant of 3.56. This finding implie a decrease in molecular weight for plasmin of 24 per cent, assuming bot molecules to have the same shape. Determination of the diffusion constan of plasminogen yields a value of 2.96×10^{-7} cm.^{2} sec.$^{-1}$. On the basis o this value, combined with the sedimentation constant, a molecular weight o

TABLE 1

PHYSICOCHEMICAL STUDIES ON HUMAN PLASMINOGEN AND PLASMIN*

Data	Plasminogen	Plasmin
Sedimentation constant	4.28	3.56
Diffusion constant (cm.^{2} sec.$^{-1}$)	2.96×10^{-7}	—
Molecular weight	141,000	107,000 (?)
Intrinsic viscosity	0.070	
Absorption spectrum—maximum	2800 Å.	2800 Å.
Tyrosine	5.1%	6.4%
Tryptophan	2.7%	3.6%
Electrophoretic mobility (pH 2.1)	8.2 and 7.3×10^{-5}	8.9×10^{-5}
Isoelectric point	6.0	7.1

* S. Shulman, N. Alkjaersig and S. Sherry; to be published.

141,000 has been calculated for plasminogen. Assuming a 24 per cent decrease in molecular size for plasmin, the latter's molecular weight may be estimated at 107,000. Viscosity studies with plasminogen have yielded a calculated intrinsic viscosity of 0.070. Assuming the plasminogen molecule to be an anhydrous elongated ellipsoid of revolution, a 7 : 1 length-to-thickness ratio may be inferred. Studying the ultraviolet light absorption spectra of these two substances, which appear to be qualitatively similar on analysis and with a single maximum at 2800 Å., Shulman has calculated that plasminogen contains 5.1 per cent tyrosine and 2.7 per cent tryptophan. Plasmin contains 6.4 per cent tyrosine and 3.6 per cent tryptophan.

On electrophoretic analysis the plasminogen preparation, at pH 2.1 in glycine buffer, ionic strength 0.10, appears to be composed of two components, producing peaks of approximately equal size, and with mobilities of 8.2 and 7.3×10^{-5}, whereas plasmin has a single peak with a mobility of 8.9×10^{-5}. At higher values of pH both plasminogen and plasmin have a single peak. It is still unclear whether the double peak seen with plasminogen at low pH represents two substances or may be associated with plasminogen itself. Since the plasminogen and plasmin preparations are poorly soluble at neutral pH, the isoelectric points must be calculated by extrapolation. From the data an isoelectric point of 6.0 has been estimated for plasminogen, and an isoelectric point of 7.1 for plasmin.

Studies on the Activation of Human Plasminogen

Spontaneous activation. Recently we have noted that plasminogen purified by the Kline technique[20] will spontaneously activate to completion in the presence of stabilizing agents.[22] Glycerin proved to be a most satisfactory stabilizing agent for plasminogen and plasmin. The plasminogen preparations were adjusted to appropriate pH and ionic strength, and were dissolved in 50 per cent glycerin. The activation velocity was dependent on the temperature, ionic strength, and pH An incubation temperature of 30° C. was found to be most suitable, for, at this temperature, no loss of plasminogen or plasmin occurred while, at higher temperatures, some loss of activity was observed. At 30° C. the activation was complete in approximately one week. The plasmin was observed to be stable for months at room temperature in the 50 per cent glycerin solution.

In FIGURE 3 are illustrated the changes taking place during the activation of a purified human plasminogen preparation in a solution containing 0.05 *M* phosphate buffer *p*H 7.6 and glycerin 50 per cent by volume, incubated at 30° C. During the activation approximately 30 per cent of the total available tyrosine of plasminogen is split off and appears as TCA-soluble material. In the particular experiment shown in FIGURE 3, human plasmin was added at the beginning of the reaction, and the activation was complete within four days. When the reaction mixture is not seeded with plasmin, the activation is slower. Our studies suggest that the activation is autocatalytic in nature. The plasmin obtained by this procedure can be stored in the glycerin solution. Otherwise, the solution can be dialyzed against 0.01 *N* HCl for removal of glycerin and salts, and then lyophilized from the acid solution. By this method we have obtained preparations of human plasmin, free of activators and of a purity similar to that of the initial "Kline" plasminogen preparation.

Urokinase activation. FIGURE 4 describes studies on the activation of human plasminogen by urokinase at several urokinase concentrations. The urokinase used in this experiment was concentrated and partially purified by the method of von Kaulla.[26] From the data in FIGURE 4 it will be noted

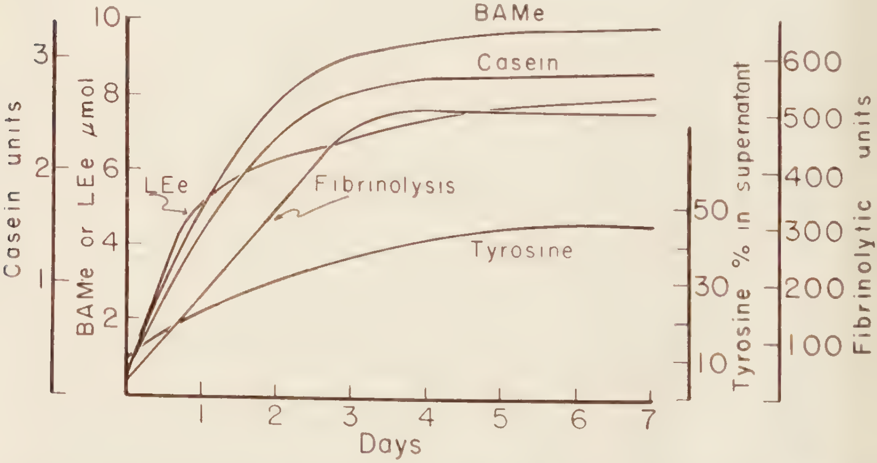

FIGURE 3. Spontaneous activation of human plasminogen in 50 per cent glycerol. Human plasminogen prepared by method of Kline.[26] See text for details of activation mixture. Methods of assay included casein proteolysis;[16] BAMe and LEe esterase activity[23] by a modification of the photometric Hestrin method;[24] fibrinolysis;[15] and release of acid-soluble tyrosine.[25] Acid soluble tyrosine release is expressed as percentage of the total available tyrosine. Fibrinolytic activity is expressed in arbitrary units: 1 unit of fibrinolytic activity represents that amount of enzyme required to lyse the standard clot in 30 min. under the conditions of the test.

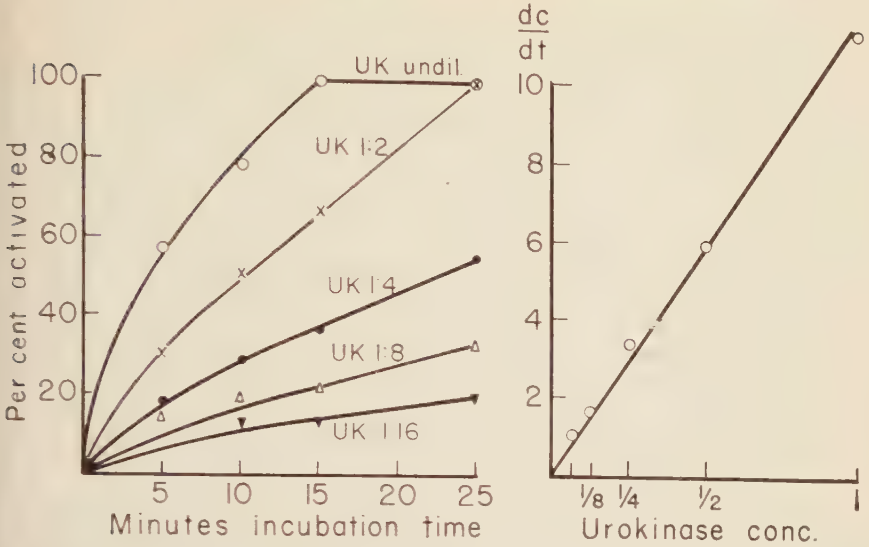

FIGURE 4. Activation of human plasminogen with urokinase (UK). Human plasminogen was prepared according to Kline.[20] Plasmin was assayed by casein proteolysis. The ordinate on the left describes the percentage of plasminogen activated. The ordinate on the right represents initial reaction velocity. Urokinase concentration is expressed as dilution of standard urokinase preparation. Incubation temperature 25° C. See text for other details.

that when the initial reaction velocities of the activation were plotted as a function of the urokinase concentration, a linear relationship was observed. Data obtained in experiments where plasminogen was varied at a fixed urokinase concentration fulfilled the criteria of a Lineweaver-Burk plot for an enzymatic reaction. These studies strongly suggest that urokinase activates plasminogen in an enzymatic or catalytic fashion.

Ploug has recently sent us a preparation of urokinase purified by chromatographic techniques.[27] This preparation is approximately fifty times more purified than the preparations of urokinase we have previously studied. Per unit of nitrogen, the preparation of urokinase prepared by Ploug is approximately one twentieth as active as the most purified streptokinase preparations presently available. In high concentrations the urokinase preparation splits esters of arginine and lysine and is proteolytic for casein. However, the esterase activity per unit of casein proteolysis is about five times greater than that observed for plasmin. The esterase activity of the urokinase appears to be intimately associated with its activating function, since the synthetic amino acid esters act as competitive inhibitors in the activation of plasminogen by urokinase.

Trypsin activation. FIGURE 5 describes studies on the activation of human plasminogen by trypsin at different trypsin concentrations. Small amounts of trypsin were utilized in these studies, so that the proteolytic activity of the trypsin would be negligible as compared to that of the plasmin formed.

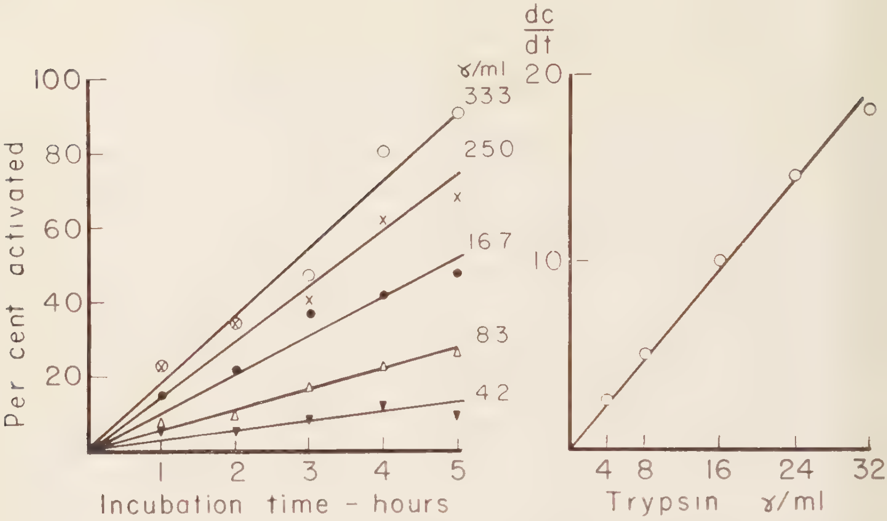

FIGURE 5. Activation of human plasminogen by trypsin. Plasminogen prepared according to Kline.[20] Plasmin assayed by casein proteolysis. Incubation temperature 37° C. Results expressed as in FIGURE 4. See text for other details.

Since the activation is relatively slow, these studies are carried out best in the presence of 50 per cent glycerin because of the latter's stabilizing effects. The linear relationship observed between activation velocity and trypsin concentration, as shown in FIGURE 5, is consistent with an enzymatic conversion of plasminogen to plasmin by trypsin.

Streptokinase activation. Streptokinase is at present the best known activator of human plasminogen. The most purified preparations of streptokinase made available to us by the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.*, contain approximately 600 units of streptokinase activity per gamma of nitrogen. A few milligamma of streptokinase can activate significant amounts of plasminogen in a matter of minutes. Since the activation of plasminogen by streptokinase is very rapid and continues while plasmin is being assayed, the activated plasmin must be separated from streptokinase in order to study the true kinetics of conversion of plasminogen to plasmin. As shown by Troll and Sherry,[25] the plasmin may be quantitatively precipitated free of streptokinase by 1 *M* NaCl at *p*H 2.0.

In FIGURE 6 is shown a study of the kinetics of plasminogen activation by streptokinase at different streptokinase concentrations. A linear relationship is noted between initial reaction velocity and streptokinase concentration. Data obtained from studies in which streptokinase concentration was kept constant and plasminogen concentration varied also fulfilled the criteria of a Lineweaver-Burk plot for an enzymatic reaction.

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* Through the courtesy of F. Ablondi, B. Hutchings, and J. Ruegsegger.

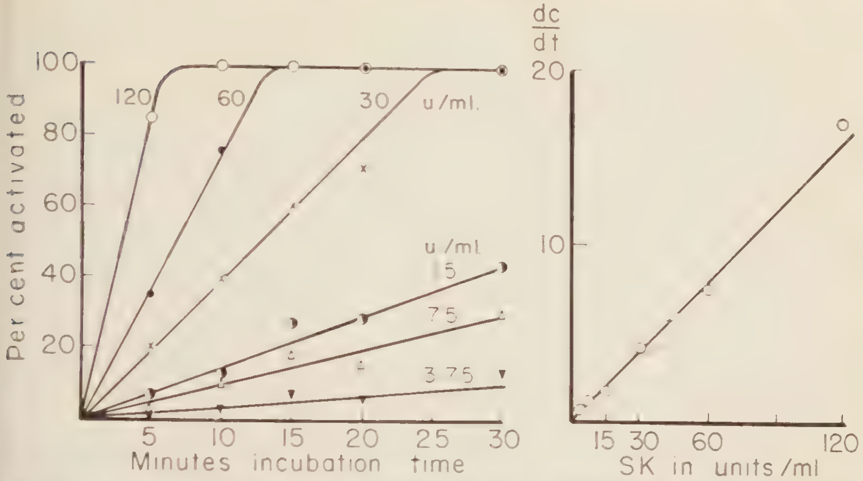

FIGURE 6. Activation of human plasminogen with streptokinase. Plasminogen prepared according to Kline.[illegible] Plasmin assayed by casein proteolysis. Incubation temperature 25° C. Results expressed as in FIGURE 4. See text for other details.

Streptokinase preparations, unlike trypsin and urokinase preparations, have never been shown to have amino acid esterase or proteolytic activity. However, in recent years, as a result of the studies of Geiger,[illegible] Müllertz,[5] and Troll and Sherry,[22] considerable evidence has been obtained suggesting that the activation of plasminogen by streptokinase is not direct, but occurs in a two-step process. In the first step, streptokinase reacts immediately, stoichiometrically and, perhaps, reversibly[illegible] with a plasma factor to form an activator. The activator then catalytically converts plasminogen to plasmin. Although the original studies of Troll and Sherry[22] demonstrated that the activator hydrolyzes lysine esters only, our more recent studies suggest that the activator formed by the interaction of streptokinase and the plasma factor splits arginine as well as lysine esters, and may even be mildly proteolytic for casein.[23]

The identity of the plasma factor that interacts with streptokinase to form an activator is still obscure. It is purified along with plasminogen or plasmin by our present procedures and has not as yet been separated from these substances. It may very well be that the activator is formed by an interaction between streptokinase and human plasmin.[19]

In all the four activations studied (spontaneous, urokinase, trypsin, and streptokinase) there appears to be a common denominator: namely, that the activation involves an enzymatic proteolytic step. All of the activations are associated with a release of fairly large amounts of acid-soluble nitrogen from plasminogen, and in each instance the activation appears to be associated with a proteolytic enzyme capable of hydrolyzing arginine and lysine esters.

The Special Fibrinolytic Activity of Streptokinase-Treated Human Plasma

We have been investigating the use of streptokinase for *in vivo* fibrinolysis for a number of reasons. Since this substance is a potent activator of the naturally occurring fibrinolytic enzyme, only small amounts of material are required; it is readily available in a fairly pure state; and its action in plasma is associated much more with *fibrinolytic* activity than with *fibrinogenolytic* or other proteolytic activity. The special fibrinolytic activity, as compared to fibrinogenolytic activity, is illustrated by the data shown in TABLE 2. For this experiment normal human plasma was incubated at 37° C. To one specimen a sample of spontaneously active human plasmin was added. At zero time an aliquot was removed and was clotted with thrombin, and the lysis time of the clot (referred to as fibrinolysis time) was determined. At frequent intervals thereafter, additional aliquots were removed and the time (referred to as the fibrinogenolysis time) was determined when no clot would form upon the addition of thrombin. To a companion specimen of human plasma, streptokinase or urokinase instead of human plasmin was added, and similar measurements were made. It can be seen from TABLE 2 that the addition of human plasmin to plasma produces a disappearance of fibrin and fibrinogen at the same rate. In contrast, the addition of streptokinase to plasma produced rapid fibrinolysis, but significant amounts of fibrinogen still remained after 60 min. Similar findings were observed when a purified urokinase preparation was added to plasma. Therefore the special fibrinolytic activity, as compared to fibrinogenolytic activity, appears to be associated with plasminogen activators rather than with plasmin itself.

There is no problem in demonstrating that plasminogen is being continuously activated in the plasma during the incubation with streptokinase or urokinase. Our data suggest the following explanation for this phenomenon: (1) plasmin arising from the activation of plasminogen in plasma is rapidly inhibited by "antiplasmin"; (2) when clotting occurs the activator is adsorbed on the clot; and (3) the plasmin activated in the interstices of the clot is not inhibited by "antiplasmin" and produces rapid fibrinolysis.

Considerable evidence supporting this explanation has been obtained, but will not be presented in this paper. Nevertheless, this special fibrinolytic activity seen in the presence of plasminogen activators seems desirable for producing controlled fibrinolysis *in vivo*.

TABLE 2
FIBRINOLYSIS VERSUS FIBRINOGENOLYSIS

Reagents	Fibrinolysis time min.	Fibrinogenolysis time min.
Human plasmin + human plasma	5	5
Streptokinase + human plasma	4	>60
Urokinase + human plasma	5	>60

The Production of Intravascular and Extravascular Fibrinolysis in Experimental Animals by Intravenously Administered Streptokinase

In experimental animals considerable success in producing intravascular fibrinolysis can be achieved with intravenous injections or infusions of streptokinase. Johnson and Tillett have demonstrated that preformed venous thrombi in rabbit ear veins can be successfully dissolved by intravenous infusions of streptokinase.[5] Studies in our laboratories have demonstrated that intravenous injections of streptokinase in dogs may result in the dissolution of experimental femoral artery thromboses.[4] A summary of these latter observations is shown in TABLE 3. From the data cited in TABLE 3, it will be noted that in 29 control animals, 3 clots spontaneously dissolved; that is, there was a dissolution rate of 10 per cent. Twenty-two animals were treated with large amounts of trypsin, that is, from 16 to 33 mg. kg. The amounts of trypsin employed produced a complete disappearance of circulating fibrinogen, yet did not influence the dissolution rate of the preformed clots. However, single intravenous injections of streptokinase on two successive days, beginning 24 hours following the production of the thrombus, increased the dissolution rate to 50 per cent. In the remaining half of the streptokinase-treated group, designated as failures, most of the clots were noted to be very soft and small and had obviously been altered.

In addition to the ability of intravenously administered streptokinase to incite the lysis of intravascular thrombi, evidence has also been obtained

TABLE 3

EFFECT OF INTRAVENOUS STREPTOKINASE ON DISSOLUTION OF INTRAVASCULAR CLOTS

Agent used	No of clots	No. of failures	No. of clots dissolved	Percentage dissolved
Control	29	26	3	10
I.V. Trypsin (16 to 33 mg. kg.) O.D. × 2	22	20	2	9
I.V. Streptokinase (250,000 units) O.D. × 2	26	13	13	50

TABLE 4

EFFECT OF INTRAVENOUS STREPTOKINASE ON PREVENTION OF POSTOPERATIVE ADHESIONS

Animals	Results			
	No. of dogs	No. with dense adhesions	No. with moderate adhesions	No. with no adhesions
Controls	21	17	4	0
Streptokinase (400,000 units) b.i.d. × 3	9	0	1	8

suggesting that extravascular deposits of fibrinous exudates can be lysed by intravenously administered streptokinase.[31] In these experiments, a traumatic fibrinous peritonitis that could be expected to proceed to organization was produced in dogs by scarifying an area of peritoneal wall with a vegetable grater. The animals were sacrificed on the 12th day and the abraded area examined grossly and histologically. Some of the data obtained in this study are summarized in TABLE 4. Of 21 control animals, 17 developed dense adhesions and 4 were observed to have moderate adhesions. Of 9 animals receiving intravenous streptokinase twice daily for 3 days beginning 24 hours after the abrasive injury, only 1 had moderate adhesions; the other 8 did not develop any.

Studies with Intravenous Streptokinase in Man

Studies with intravenous streptokinase in man were begun by Tillett and his group several years ago, and many of the basic principles involved in the intravascular use of streptokinase were described in detail[14] in 1955. These exploratory studies with streptokinase intravenously administered in man have until recently been concerned primarily with the activation of plasminogen *in vivo* and with the toxicity encountered. With the availability of more purified preparations of streptokinase, studies have now progressed into the second phase, to determine whether intravascular streptokinase is an effective therapeutic agent.

Since the more extensive experience of Tillett and Johnson with intravascular streptokinase is summarized elsewhere in this publication,[32] our observations will be summarized briefly. To date we have administered 104 intravenous infusions of streptokinase to 36 patients. The dose required to set up fibrinolytic activity *in vivo* was estimated for each patient by a preliminary *in vitro* test in which 1 ml. aliquots of the patient's plasma were mixed with serial dilutions of streptokinase and then clotted with thrombin. The tubes were incubated at 37° C. and the lysis time of each of the specimens recorded. Arbitrarily, the dose of streptokinase to be used *in vivo* was determined by multiplying the estimated plasma volume of the patient by the amount of streptokinase that produced a lysis time of approximately 10 min. in the *in vitro* test. The total estimated dose was then infused over a 2- to 3-hour period. In approximately 50 per cent of the infusions, specimens of blood removed from the patients during the latter part of the infusion showed fibrinolytic activity. The specimens clotted normally and, when incubated at 37° C., lysed completely over the next 1 to 4 hours. More rapid fibrinolytic activity could be achieved by speeding the infusion or by raising the dose. In those instances in which no fibrinolytic activity was observed, the dose was usually raised by increments of 25,000 to 50,000 units on subsequent days. With these techniques, circulating fibrinolytic activity was achieved in approximately 75 per cent of the patients studied.

In FIGURE 7 are shown representative biochemical data obtained from a patient who received an infusion of 75,000 units of streptokinase over a 2-hour period. It should be noted that the fibrinolytic activity of the patient's blood rose rapidly at the onset of the streptokinase infusion, reached a maximal level at the end of the infusion, and then fell off rapidly. Plasminogen activation was demonstrable during the infusion, but ceased at the end of the infusion. Normal plasminogen levels were restored over the next 3 days. Only small amounts of circulating proteolytic activity could be demonstrated at any time thereafter and are not shown in FIGURE 7. The fibrinogen concentration fell to about half the normal during the infusion and returned to normal over the next 48 hours. The antiplasmin activity of the plasma fell during the period of plasminogen activation, but promptly returned to the pretreatment level with the cessation of streptokinase administration.

We have not observed allergic reactions to the streptokinase infusions. Chills and fever occurred with some regularity as a side effect of the cruder preparations of streptokinase originally used. In the last 73 infusions with more purified preparations of streptokinase, made available to us by the

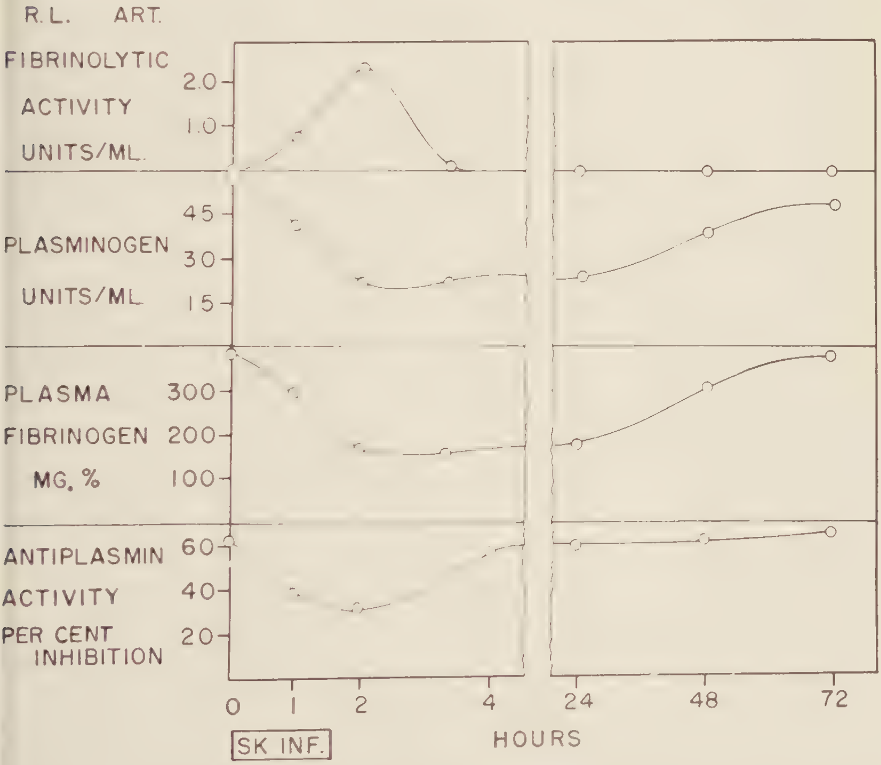

FIGURE 7. Effects of an intravenous streptokinase infusion in a patient with arteriosclerosis. Infusion of 75,000 units of streptokinase in 250 ml. of 0.85 per cent NaCl was given over a 2-hour period. Fibrinolytic activity was determined by measuring the whole blood clot lysis time and is expressed in arbitrary units (1 unit equals lysis time of 30 min.). Plasminogen assayed by BAMe esterase method;[23] fibrinogen by the method of Ratnoff and Menzie.[33] Antiplasmin activity is expressed as the percentage of inhibition by 0.2 ml. plasma of a standard bovine plasmin preparation after 15 min. incubation at 25° C.

Lederle Laboratories, we have seen only an occasional chill and there has been a marked decrease in the incidence of pyrogenic reactions. Little, i any, hypotension has been observed.

On 6 occasions bleeding that might well be attributed to the streptokinase infusion was noted. In 3 instances, twice from the gums and once from a previously abraded area on the face, a bloody ooze appeared *during* the streptokinase infusion and at the height of circulating fibrinolytic activity The bleeding spontaneously ceased shortly after the infusion was discontinued. In 3 other patients, bleeding was observed *after* the streptokinase infusion was completed. One patient developed a subcutaneous hematoma at a pressure site, another suffered bleeding from the nasopharynx around an encrusted nasogastric tube, and the third patient coughed up some blood-tinged sputum. These episodes were of transient nature, were not serious and spontaneously ceased. Estimations of capillary fragility and of bleeding and clotting times were normal. No other serious toxic manifestations have been noted.

During the past 4 months, using more purified preparations of streptokinase, we have treated 23 patients with intravenous streptokinase. These patients have each received from 1 to 6 infusions of streptokinase, in doses of from 50,000 to 350,000 units per infusion. In the total of 73 infusions as previously mentioned, only a minor incidence of undesirable pyrogenic reactions was noted.

The diagnoses of these patients are shown in TABLE 5. There were 11 patients with acute thrombophlebitis, and 2 of these had recurrent pulmonary emboli. One patient had a chronic cellulitis of the left leg. There were 4 patients with cerebrovascular accidents, 3 thrombotic and 1 embolic. There was 1 patient with a cerebrospinal block secondary to a chronic arachnoiditis of unknown etiology. Two patients had auricular fibrillation secondary to rheumatic heart disease. They were treated with streptokinase before conversion of their arrhythmia with quinidine. The 2 patients with suspected subacute bacterial endocarditis had persistent fever and cardiac murmurs, but had negative blood cultures. The streptokinase was used in these patients as a provocative agent. Finally, there were

TABLE 5
DIAGNOSES IN 23 PATIENTS "TREATED" WITH INTRAVENOUS STREPTOKINASE

No. of patients	Diagnosis
11	Acute thrombophlebitis
1	Chronic cellulitis
4	Cerebral vascular accident
1	Chronic arachnoiditis
2	Auricular fibrillation (rheumatic heart disease)
2	? Subacute bacterial endocarditis
1	Popliteal artery thrombosis
1	Chronic organizing pneumonia

single instances of a popliteal artery thrombosis and a chronic organizing pneumonia.

Most of the patients with thrombophlebitis and the patient with chronic cellulitis showed an improvement during and immediately following the streptokinase infusions. The responses ranged from a gradual to a fairly dramatic subsidence in the acute inflammation. In several instances a palpable phlebitic cord rapidly disappeared. In the 2 patients with recurrent pulmonary emboli, no further embolization occurred. We did not observe improvement in the cases with cerebral thrombosis, but therapy in each instance had been initiated more than 24 hours after the onset of neurological change. In the patient with chronic arachnoiditis, the cerebrospinal block disappeared rapidly even though no improvement in the general condition of the patient was observed. Despite the beneficial effects observed in many of these patients, we do not as yet have specific evidence that intravascular or extravascular fibrinolysis occurred in any of the patients. The fairly dramatic changes observed in several instances may still be consistent with the variations seen in the natural course of disease or may simply be associated with the anti-inflammatory effect that may follow injections of a variety of proteolytic enzymes; this is discussed in detail in other papers in this monograph. However, we have been encouraged by these preliminary observations and feel that there is considerable justification for expanding them at the level of clinical investigation. Nevertheless, at this time one cannot overemphasize the need for continued caution both in the use of these agents and in the interpretation of the results.

Summary

Studies have been presented on some characteristics of the fibrinolytic enzyme of human blood (plasmin or fibrinolysin), on the mechanism of activation of plasminogen, and on the experimental dissolution of intravascular thrombi and extravascular fibrinous exudates in dogs by intravenous streptokinase. Exploratory observations on the application of this information for therapeutic use in man have been described briefly.

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PROTEASES IN THE CLOTTING MECHANISM*

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A number of substances participate in the reactions that take place when blood clots. These substances may interact consecutively or simultaneously. Although many of the components are characterized incompletely, rapid progress is being made in this direction. The concept of a system of proteolytic enzymes in action during coagulation of blood is evolving slowly.

The blood-clotting system may be visualized as a series of specific hydrolytic enzyme reactions and stoichiometric interactions between the various clotting factors. Accumulated investigations have demonstrated the presence of a versatile precursor and an active enzyme having polyvalent properties.[1]

Thrombin originates from prothrombin by a process of degradation that is now known to proceed by various pathways.[1]

Physiologically, prothrombin is degraded to thrombin by a proteolytic enzyme complex the activity of which is governed by an inhibitor. This complex, designated as a "prothrombinase"[2] is made up of at least three essential components: Ac-globulin, thromboplastin, and calcium ions in proper proportions. Variations in concentration of any one of these three essential units result in variable thrombin yield. The definition of such a tripartite enzyme deviates somewhat from the usual conventional interpretations.

The inhibitor in this case is defined as the "heparin-cofactor complex," which is presumed to be the result of the interaction of heparin or heparinlike substances with a cofactor. To date, evidence based upon human subjects indicates that the physiologically occurring heparinlike complex quantitatively inactivates Ac-globulin and does not alter prothrombin. Heparin-cofactor complex affects the activity of "prothrombinase" by inhibiting one of the essential pieces of the enzyme complex, that is, Ac-globulin. Under these conditions blood will not clot. Ac-globulin deficiency as an etiological factor in hemorrhagic diathesis has been reported in the literature.

In the dog, the situation is somewhat different. Ac-globulin and prothrombin are inactivated simultaneously by the heparin-cofactor complex. The significance of such species specificity is not clear at the present time. It is well known that intravascular clotting in the dog is rare and that it is difficult to induce experimentally. One may conjecture that such behavior is a manifestation of the strong homeostatic mechanism in this animal.

Autocatalytic conversion[3] of purified prothrombin to thrombin, in which intermediate accelerators and activators are not necessary, has also been demonstrated. This implies that prothrombin is a true precursor, possessing the potentiality of spontaneously metamorphosing to the active enzyme.

Proteolytic enzymes, for example, trypsin,[4] convert prothrombin to

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thrombin. This conversion appears to be an enzymatic reaction of the proteolytic type. The foregoing observations illustrate the role of proteases in the first phase of blood coagulation. Regardless of the nature of the hydrolytic enzyme, the end result is the same; namely, thrombin is produced as one of the products.

Investigation has revealed that thrombin has multiple properties: (1) It converts fibrinogen (sol) to fibrin (gel), a phenomenon that has been observed for many years. This property places the clotting component in the category of polymerases. (2) It splits off fibrinopeptide;[5] this places it in the category of the proteases. (3) It hydrolyzes a specific synthetic substrate, tosyl arginine methyl ester,[6] indicating that it can act as an esterase. (4) It lyses fibrin clots,[7, 8] and thus manifests some of the characteristics of a fibrinolysin. In addition to being a proteolytic enzyme, thrombin is a versatile blood clotting component. The diverse properties of this agent are being explored in great detail.[1]

In preliminary studies with thrombin as a fibrinolysin, the resulting products have been found to be electrophoretically different from those obtained with trypsin and plasmin.[9]

Using the fibrin-plate method[10] we obtained no lysis with purified prothrombin.* However, fibrin dissolution was observed after 65 hours of incubation. The fluid portion now contained active thrombin. Thus, it was after the spontaneous conversion of prothrombin to thrombin on the fibrin plate that liquefaction of the clot occurred. This experiment suggests the relationship of fibrinolysis to the appearance of thrombin.

Thrombin can be given intravenously to animals by slow drip without defibrinogenation or the appearance of fibrin in the lungs. One hundred units were given intravenously to 30 dogs during 1 hour by continuous drip without intravascular complications. These animals recovered uneventfully. They were subsequently sacrificed, and no evidence of intravascular clotting was found. This observation may indicate that the antithrombin mechanism compensated by rapid inactivation of the clotting component. On the other hand, when 100 units were administered intravenously in 5 minutes, the entire vascular tree was clotted, resulting in instant death of the animal.

Clinical experiences in the human subject with intravenous thrombin in conjunction with coumarin anticoagulant therapy for thrombotic complications has yielded encouraging results.[11] We selected subjects who showed no response to the anticoagulant drugs notwithstanding drastic reduction in the clotting potential of the circulating blood. Slow intravenous administration of 100 units of thrombin (45 minutes by continuous drip) elicited an objective clinical response in a group of 10 patients. The number of cases is too small to draw far-reaching conclusions. It may be conjectured, however, in view of laboratory data regarding the fibrinolytic properties of thrombin and of the possible homeostatic compensation of the antithrombin mechanism, that the associated symptoms of the vascular occlusion were alleviated. Extreme caution must be exercised, and the possible catastrophic consequences must be kept foremost in mind if such heroic therapy is elected as a last resort. It is of special interest to note that relief from thrombotic sequelae may be obtained in unusual clinical cases when thrombin administered intravenously is used in conjunction with coumarin anticoagulants. This is not a recommended procedure at present, although no side effects whatsoever have been observed in the cases studied. The theoretical implications, however, are legion.

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* This preparation was kindly supplied by W. H. Seegers, Wayne University College of Medicine, Detroit, Mich.

In conclusion, the blood-clotting mechanism thus presents a dynamic enzymatic picture: stoichiometric interaction between polyvalent components; and proteolytic degradation of an inactive precursor to give rise to an active protease with esterase properties. Furthermore, this enzyme has the property of polymerizing a specific protein (fibrinogen) to a gel by partial degradation and, in turn, by continued lytic action, to a sol.

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Discussion of the Paper

Question: I should like to ask whether protamine destroys Ac-globulin in plasma that is not previously defibrinated.

C. E. Brambel: It destroys Ac-globulin, whether the plasma is defibrinated or not. However, the effect of protamine is greatest in plasma before it is defibrinated. It has a weaker effect in defibrinated plasma, stopping the progressive destruction of Ac-globulin.

J. L. Ambrus (*Roswell Park Memorial Institute, Buffalo, N. Y.*): There are many reports claiming that thrombin itself is a fibrinolytic agent, which might account for your results independently of contamination.

C. E. Brambel: I think Bordet called attention to the fibrinolytic properties of thrombin; furthermore, in his Harvey Lecture W. H. Seegers pointed out the lytic properties of thrombin. At any rate, the possibility that our preparations were contaminated must be taken into account.

S. Sherry (*Jewish Hospital, St. Louis, Mo.*): There is very little doubt that thrombin exhibits tremendous *fibrinogenolytic* activity but the question remains whether it has a little fibrinolytic activity or none at all. I think that at the present moment the question is whether Brambel's materials are contaminated with plasmin. It is conceivable that thrombin has a little fibrinolytic activity but, relative to its activity upon fibrinogen, this is very small indeed.

ANTI-INFLAMMATORY EFFECT OF TRYPSIN

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A dictionary defines "concept" as "a thought or an opinion." When the question "How does trypsin act in inflammation?" is asked, we must concede that the mode or mechanism of action is unknown. However, we offer the following concept.

Localization of tissue damage, a cardinal aspect of the inflammatory process, is in part due to fibrin deposition, with the consequent formation of a mechanical barrier in the lymphatics and in the tissue spaces. This concept of Menkin (1950) has been extended by Day (1954) to include connective tissue and modifications in its permeability due to deposition of fibrin in its fine mesh structure. The protein fine structure, which is macromolecular in dimension, undergoes modification of permeability as macromolecules are caught in the network, causing some impaction of its pores. It is our proposal to extend this concept to capillaries, lymphatics, and even to cellular elements.

Permeability of the capillary is therefore related to the pore size of the network and to the degree of blockage of these pores by macromolecules. The macromolecular units could be any polymerizing material, for example, protein, hyaluronic acid, or nucleic acid. Assuming the existence of the basic protein network, the prime factors controlling permeability would be any influences or factors modifying the pore size or modifying the amount of macromolecular material impacted in the network.

The initial inflammatory reaction relates primarily to the production, in a manner presently unknown, of a chemical that modifies the pore size of the protein mesh. It is our belief that this chemical is either a peptide or a modified protein and that the formation of this unit is related to cellular disruption followed by cathepsin action on the recently liberated cellular content. Spector (1951) has isolated from inflammatory tissue a series of peptides capable of producing *in vivo* the reactions associated with inflammation.

Another possibility is that the inflammatory peptides of Spector (1951) may function as inhibitors of trypsin and therefore, in all probability, of all proteolytic enzymes, including plasmin. If the inflammatory peptide inhibited plasmin locally, deposition of fibrin in the protein network of the capillary wall would not be counteracted by a fibrinolytic action. Thus, these peptides would not only increase porosity, but would also tend to inhibit the process that removes one macromolecular form, fibrin, from the network.

Modification of the pore size permits passage into the tissue spaces of water, small molecular units, and macromolecules. Fibrinogen passing through the protein network is converted into a labile form of fibrin, and this is trapped in the network. The result is blockage or trapping of the intercellular fluid. The hydrostatic pressure exerted by the trapped intercellular fluid mechanically closes many capillaries and lymphatics. Mechanical closure creates a physical situation in which the approximated surfaces of the capillary form the focal point for additional fibrin formation. The walling-off process is now essentially complete. Biological continuity is eliminated.

The fibrin, whether in the network of the capillary wall or in the capillary and lymphatic space, is in a particularly labile form. In other words, the degree of polymerization of this fibrin does not make it comparable to the fibrin of a fixed, formed clot. The fibrin deposit is not a clot in the customary sense of the word; it is rather a gel-sol point, a state representing the knife edge of balance between the solid and the colloidal forms. A shift in the equilibrium of this physical form can be caused in many ways, but the one operative here is probably related to proteolytic enzymes.

Trypsin administered intramuscularly or intravenously in therapeutic dosages acts at the inflammatory site. The concept of plasmin activation at the site of injection no longer seems tenable since no detectable differences in blood plasmin concentrations have been found. At the site of the inflammation, trypsin functions as a depolymerase. It may also cause some plasmin activation. In any event, it does not seem improbable that fibrin concentrates, by adsorption, the depolymerizing proteolytic enzymes. Müllertz (1953) has clearly demonstrated the strong and specific adsorption of plasmin to fibrin. Extrapolation of these findings leads to the conclusion that adsorption of all proteolytic factors may occur. This localized concentration of enzyme reverses the fibrinogen-fibrin reaction; the macromolecules are removed from the protein network wall of the capillary. The porosity is then increased and the intercellular fluid is released. Hydrostatic pressures are reduced; capillaries open; biological continuity is restored; and circulation in the local area is restored. With that restoration the multiple defensive forces of the blood are brought into play and all soft fibrin deposits are removed.

The key, then, to the action of trypsin in an inflammatory reaction is directly related to the restoration of biological continuity and circulation, and thereby to the prevention of further cellular damage and death. The inflamed area does not become necrotic and no abscess formation occurs.

The highly complex coagulation and fibrinolytic mechanisms operative *in vivo* emphasize the scope of application of biological antagonism. In this system, antagonistic forces of the greatest complexity wage the constant warfare essential to the maintenance of homeostasis and, therefore, of life. The role of medicine is restoration of the delicate balances of biological antagonism.

Two major biological concepts govern every aspect of life: biological antagonism and biological relativity. One might state that these two laws constitute the warp and the woof of the life pattern. Physics has long had its monumental basic concepts; biology has had very few, but now these are emerging, and with that emergence will come clarification. The fundamentals of the action of trypsin *in vivo* relate to an aspect of biological relativity, properly designated "differential kinetics and dynamics." The relative speed of action of enzyme systems in the enzymatic concatenation of the living cell is a determinant of normality.

If this concept is to have any meaning, quantitative evaluation is necessary. The first approach to a resolution of the problem was an investigation of the effects of various enzymes on the edema induced by egg white (TABLE 1). In our hands this technique has proved susceptible to a considerable degree of quantitation. As with all biological procedures, the value of the technique depends to a large extent on the skill of the technician, and a study of egg-white edema requires considerable experience.

The results show that all the enzymes tested were effective in inhibiting the formation of edema. However, the effects appear to bear no relationship

TABLE 1
INHIBITION OF EGG-WHITE EDEMA BY PROTEOLYTIC ENZYMES

Enzyme	Dose (mg./kg.)	Wt. difference, right leg − left leg (gm.)	Standard deviation	Inhibition (%)
C*....		1.50	.06	
Trypsin	10	0.80	.18	45
C	—	1.38	.08	—
Trypsin	2	0.90	.05	35
C	—	1.48	.07	—
Trypsin	1	1.40	.18	0
C	—	1.68	.13	—
Streptokinase†	20	1.30	.12	20
C	—	1.68	.13	—
Streptokinase	10	1.40	.12	15
C	—	1.63	.23	—
Streptokinase	2	1.50	.27	0
C	—	1.90	.09	—
Chymotrypsin	2	0.93	.20	50
C	—	1.90	.09	—
Chymotrypsin	1	1.83	.20	0
C	—	1.68	.13	—
Prolase B	10	1.13	.15	30
C	—	1.90	.09	
Prolase B	2	1.51	.19	20
C	—	1.90	.09	—
Prolase B	1	1.75	.21	0
C	—	1.97	.17	—
Fibrinolysin‡	400	1.70	.12	15
C	—	1.93	.08	—
Fibrinolysin	200	1.93	.11	0

* Control.

† The commercial preparation Varidase was used.

‡ Obtained by courtesy of E. C. Loomis, of Parke, Davis & Company, Detroit, Mich.

to ability to catalyze the profibrinolysin-fibrinolysin reaction. Thus chymotrypsin, which does not catalyze this reaction (Mertz and Jackson, 1954) is of the same order of activity as trypsin; the effect of fibrinolysin, even at the very high doses used, is of doubtful significance. The fact that an effect was shown by several different enzymes suggested that it might be a function of simple proteolytic activity. In order to investigate this possibility, partially and completely inactivated samples of trypsin were tested for their effect on egg-white edema. Proteolytic activity was determined by the method of Anson (1938).

For standard crystalline trypsin, at 100 per cent activity, the minimum effective dose (MED) was 2 mg./kg. (see TABLE 1). For trypsin at 50 per cent activity, the MED was 4 mg./kg; completely inactivated trypsin showed no anti-inflammatory effect in doses up to 10 mg./kg. This finding shows definitely that the anti-inflammatory effect of trypsin is a function of its proteolytic activity. Although the action of the profibrinolysin-fibrinolysin system is not precluded, these results show that anti-inflammatory effects are manifested by enzymes that do not catalyze this reaction. Moreover, fibrinolysin itself has a comparatively weak action. These findings have been confirmed by Adamkiewicz *et al.* (1955) for local edema produced by egg white, kaolin, and yeast filtrate in normal and in adrenalectomized rats. Trypsin also has been reported to inhibit the generalized edema produced by dextran (Cohen *et al.*, 1955). On the other hand, Hardy *et al.* (1955) observed no effect of a trypsin-in-oil preparation on local edema induced by dextran in adrenalectomized rats.

It is most interesting to note the differences in sensitivity to trypsin manifested by edemas induced by different phlogistic agents. TABLE 2 gives the

TABLE 2
EFFECT OF TRYPSIN ON EDEMA PRODUCED BY VARIOUS PHLOGISTIC AGENTS

Agent	Dose trypsin (mg./kg.)	Administration	Wt. diff. (saline leg − edema leg) (gm.)		Inhibition (%)
			Control	Experimental	
Yeast	5	S.C.	1.35 ± .10	1.00 ± .10	25
Dextran	5*	S.C.	1.26 ± .14	1.14 ± .17	0
	20*		1.26 ± .14	0.98 ± .14	25
	5		1.26 ± .18	1.26 ± .05	0
	20		1.26 ± .18	0.76 ± .17	40
	5*	I.P.	1.20 ± .10	1.34 ± .23	0
	20*		1.20 ± .10	0.88 ± .15	20
	5		1.20 ± .10	0.80 ± .12	25
	20		1.26 ± .14	0.66 ± .21	45

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* Trypsin-in-oil preparation (Parenzyme, The National Drug Co., Philadelphia, Pa.) was used.

results obtained on local administration of 2 different phlogistic agents. Obviously the edemas produced by these agents are not equivalent as far as their inhibition by trypsin is concerned. Egg-white edema is inhibited by trypsin (Martin *et al.*, 1954) at a concentration of 2 mg./kg., but the dextran edema does not respond to trypsin in either water or oil on subcutaneous administration at a level of 5 mg./kg. The edema produced by yeast is apparently quite sensitive. Adamkiewicz *et al.* (1955) have reported its inhibition by a total dose of a trypsin-in-oil preparation amounting to 0.81 mg./kg.

The increase in effect obtained with the aqueous trypsin on intraperitoneal administration suggested that speed of absorption might be a factor affecting the results; this is undoubtedly of prime importance in an acute experiment. A number of animals were treated with trypsin at varying intervals before the production of a dextran edema in order to determine whether the half-hour interval routinely used allowed sufficient time for the development of an optimal blood level. This was felt to be of particular interest with regard to the trypsin-in-oil preparation, with which some delay in absorption might reasonably be expected to occur. The results from this experiment are presented in TABLE 3. All doses of trypsin were given subcutaneously. The figures show that time of administration is definitely a factor in the results. Both preparations used show no effect at the 5-mg. level when given one half hour before edema development, but give a definite effect when a 3-hour interval is allowed to elapse. The effect of the 20-mg. doses is at a high level after 24 hours; 5-mg. doses show only a slight effect at this time interval and, presumably, the peak effect has been passed somewhere between 3 and

Table 3
Effect of Time of Administration on the Action of Trypsin on Local Dextran Edema

Time (hr.) of administration (before edema production)	Dose (mg./kg.)	Wt. diff. (saline leg − dextran leg) (mg.)		Inhibition (%)
		Control	Experimental	
0.5	5*	1.26 ± .14	1.14 ± .17	0
0.5	20*	1.26 ± .14	0.98 ± .14	25
0.5	5	1.26 ± .18	1.26 ± .05	0
0.5	20	1.26 ± .18	0.76 ± .17	40
3	5*	1.14 ± .05	0.96 ± .15	15
3	20*	1.14 ± .05	0.88 ± .06	25
3	5	1.14 ± .05	0.82 ± .06	30
3	20	1.14 ± .05	0.62 ± .18	45
24	5*	1.46 ± .17	1.18 ± .12	20
24	20*	1.26 ± .05	0.67 ± .13	45
24	5	1.20 ± .10	0.90 ± .20	25
24	20	1.26 ± .05	0.52 ± .08	60

* Trypsin-in-oil preparation (Parenzyme) was used.

24 hours. One may conclude that the assumption that a system is saturated with trypsin at an arbitrarily chosen dosage schedule may be unjustified in a given experimental technique.

In TABLE 4 are presented results obtained with generalized edemas produced by intraperitoneal injection of egg white and dextran. In all cases the trypsin was injected subcutaneously, and the observations were made 3 hours after injection of the phlogistic agent, when edema was invariably well marked in the extremities of all the controls. It is apparent that a higher dose of trypsin is required to inhibit an edema of this type than is necessary for a purely local edema. This observation again emphasizes the fact that experimentally produced edemas of different types may have quantitatively different responses to trypsin. The results obtained with the generalized dextran edema confirm work done by Cohen *et al.* (1955). These workers noticed no difference in the response of normal, adrenalectomized, or hypophysectomized rats in this phenomenon. Adamkiewicz *et al.* (1955) also found no difference in response to trypsin of adrenalectomized rats with local egg-white edema.

The findings as a whole indicate that trypsin is effective in inhibiting several different types of experimentally produced edema. They also show that the different types of edema are not necessarily equivalent quantitatively in their response to trypsin and, therefore, that dosages and dosage schedules that have been found to inhibit one type of edema will not necessarily prove effective against another. Thus, the negative results obtained by Hardy *et al.* (1955) may be explained on the basis of insufficient dosage. This consideration obviously applies with at least equal force to clinical edemas as opposed to those produced in the laboratory.

As stated, the evidence indicates that the inhibitory action of trypsin in egg-white edema is not primarily concerned with the catalysis of the conversion of profibrinolysin to fibrinolysin. This finding raised the question as to whether some other indirect mechanism is concerned or whether the enzyme acts directly to facilitate drainage at the site of edema. Results suggest that trypsin has a direct mode of action, and that its anti-inflamma-

TABLE 4
INHIBITION OF GENERALIZED EDEMA BY TRYPSIN

Phlogistic agent	Dose trypsin (mg./kg.)	Inhibition of edema (%)
Egg white	10	0
	20	25
	40	50
	50	75
Dextran	5	0
	10	0
	20	50
	50	100

TABLE 5

EFFECT ON EGG-WHITE EDEMA OF TISSUES PREINCUBATED WITH TRYPSIN

Tissue	Weight difference, saline leg − egg-white leg (gm.)	Inhibition (%)
Liver	1.92 ± .07	0
Control	2.15 ± .12	
Kidney	2.05 ± .07	0
Control	2.15 ± .12	
Spleen	2.12 ± .12	0
Control	2.15 ± .12	
Muscle	1.92 ± .07	0
Control	2.15 ± .12	
Blood	1.42 ± .07	30
Control	2.12 ± .11	
Blood & trypsin*	1.60 ± .05	25
Control	2.12 ± .11	
Plasma	0.76 ± .04	45
Control	1.42 ± .14	
Plasma & trypsin*	1.35 ± .12	0
Control	1.42 ± .14	
Serum	1.50 ± .15	20
Control	1.95 ± .12	
Serum & trypsin*	1.70 ± .15	0
Control	1.95 ± .12	

* Dosage 0.6 ml. in 2 doses one-half hour apart throughout.

tory effect is the result of a combination of several different physiological actions.

The results reported in TABLE 5 are averages for groups of five animals; each experimental group is run with its own control. The figures show that in no case was inhibition of edema obtained using tissue homogenates preincubated with trypsin. Blood, plasma, and serum caused some inhibition, suggesting the presence of an anti-inflammatory factor, but in no case was this effect heightened by trypsin pretreatment; indeed, it appeared to be counteracted. The negative results obtained with these trypsin-treated tissues do not, of course, exclude the possibility that in the body trypsin acts on some tissue to produce proteolytic products that in turn modify the experimental edema. However, the figures presented in TABLE 6 show that trypsin, when injected with the egg white directly into the site of edema, is effective at an appreciably lower dosage than when it is administered at other points.

The most logical interpretation of this finding is that trypsin acts directly on the edema without any intermediate reaction, and that administration at the site of edema simply provides a high local concentration at the point where the enzyme acts. It is worthy of mention that the presence of a specific trypsin inhibitor in the egg white may affect the activity of the enzyme when it is administered in this fashion, so that the figures may be taken as representing a minimal effect.

TABLE 6
EFFECT OF PHYSIOLOGICALLY ACTIVE AGENTS ON EGG-WHITE EDEMA

Agent	Dose	Wt. difference, saline leg − egg-white leg (gm.)	Inhibition (%)
Trypsin	1 mg. kg. (1 dose S.C.)	1.77 ± .12	0
Control		1.80 ± .05	
Trypsin	1 mg. kg. (1 dose with egg white)	1.35 ± .15	30
Control		1.90 + .05	
Hyaluronidase	400 TRU (with egg white)	1.55 ± .17	25
Control		2.05 ± .12	
Hyaluronic acid	10 mg. kg. (with egg white)	1.65 ± .12	0
Control		1.80 ± .05	
Hyaluronic acid (partially depolymerized)	10 mg. kg. (with egg white)	1.35 ± .20	25
Control		1.80 ± .05	
Mecholyl	2.0 mg. kg. (1 dose S.C.)	0.75 ± .25	60
Control		1.85 ± .05	
Heparin	3000 units kg. (1 dose I.P.)	1.80 ± .10	0
Control		1.94 ± .15	
Trypsin	1 mg. kg. (2 doses S.C.)	1.40 ± .18	0
Control		1.48 ± .07	
Chymotrypsin	1 mg. kg. (2 doses S.C.)	1.85 ± .20	0
Control		1.90 ± .09	
Trypsin & chymotrypsin	1 mg. kg. each (2 doses S.C.)	1.30 ± .25	35
Control		2.02 ± .15	

TABLE 6 gives the results obtained with several compounds that were tested because of similarities in their physiological action to that of trypsin. Hyaluronidase has been reported (Glassman *et al.*, 1953) to inhibit the inflammatory reaction by causing an increase in vascular permeability and thus facilitating drainage. Partially depolymerized hyaluronic acid has also been reported to increase permeability (Seifter and Baeder, 1954). Both of these substances were effective in inhibiting egg-white edema. Both will, of course, facilitate intradermal dye spreading. Trypsin, as well as other proteolytic enzymes that inhibit this edema (Martin *et al.*, 1954), also has such an effect; proteolytic enzymes inactive against egg-white edema have no effect on interdermal dye spreading (Beiler and Martin, 1955).

These findings suggested that the anti-inflammatory effect of proteolytic enzymes might be due in part to their effect on tissue permeability. Trypsin was found to have no effect on the permeability of isolated connective tissue. However, when the permeability of the tissue was reduced by treatment with a mixture of plasma and thrombin (FIGURE 1), addition of trypsin tended to restore it to normal, indicating the removal of fibrin deposits from the pores of the tissue. This finding is suggestive in view of our concept of the role of fibrin deposition in the inflammatory process. *In vitro* trypsin and other proteolytic enzymes were found to enhance intracutaneous dye spread-

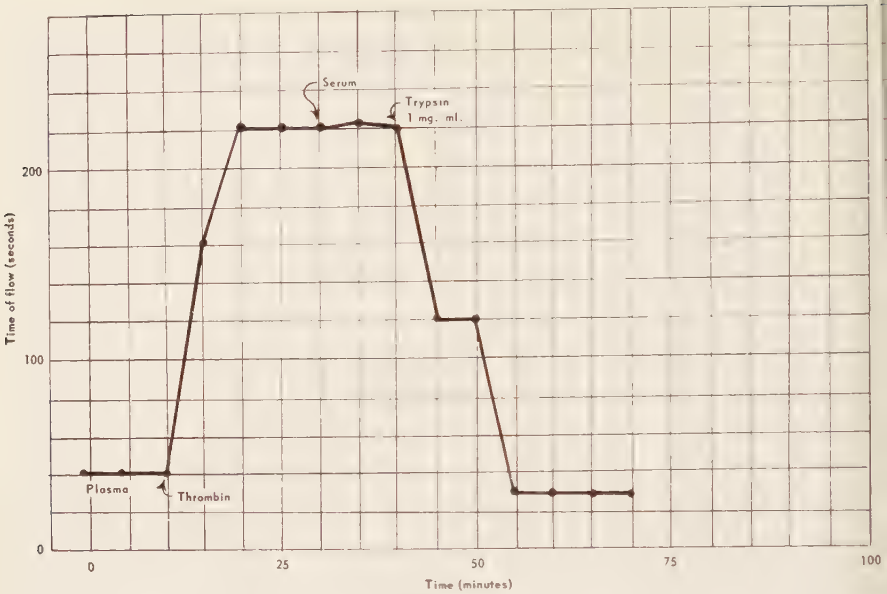

FIGURE 1

ing in the rabbit, indicating an increased tissue permeability (TABLE 7). The increase in area of spread varied with the anti-inflammatory effect of the enzyme tested; thus plasmin, which had no appreciable anti-inflammatory action, caused no increase in intracutaneous dye spreading.

Trypsin causes vasodilatation (Rocha e Silva, 1940). Mecholyl, a potent vasodilator, had a pronounced effect on the experimental edema (TABLE 6). On the other hand, heparin, administered one-half hour before the production of edema in sufficient concentration to render the blood incoagulable during the course of the experiment, was without inhibitory effect. A combination of trypsin and chymotrypsin at concentrations of each enzyme that individually had no effect was markedly inhibitory. These two enzymes are

TABLE 7
EFFECT OF PROTEOLYTIC ENZYMES ON INTRACUTANEOUS DYE SPREADING IN RABBITS

Enzyme	Dose	Increase in area of spread (%)	Inhibition of egg-white edema
Hyaluronidase	0.2 mg.	25	+
Prolase B	0.2 mg.	25	+
Chymotrypsin	0.2 mg.	50	+
Trypsin	0.2 mg.	60	+
Trypsin	20 mg. kg. I.P.	100	+
Plasmin	0.2 mg.	0	—
Saline	—	0	—

known to reinforce each other's proteolytic action (Northrop *et al.*, 1948), and the effectiveness of the combination in inhibiting the development of egg-white edema may be related to the observation that trypsin produces a decrease in viscosity of the body fluids (Innerfield *et al.*, 1953).

Thus, it would appear that the anti-inflammatory action of trypsin is a result of a direct facilitation of drainage from the inflamed area. The capacities of trypsin to increase permeability, to cause vasodilatation, and to reduce the viscosity of edema fluid will all have the effect of facilitating drainage. Agents that have these properties individually will inhibit the experimental edema.

An investigational pattern to support the concept of direct action by trypsin relates to the general concept of biological antagonism; therefore, studies were undertaken to determine the role of serum inhibitor in the antiphlogistic action of trypsin. Sherry *et al.* (1952) observed incomplete neutralization of trypsin by large excesses of trypsin inhibitor *in vivo*. They reported that in undiluted plasma combination between trypsin and trypsin inhibitor was not stoichiometric (Sherry *et al.*, 1954). Bier *et al.* (1955) suggested that trypsin derivatives having less combining power with the serum inhibitor might be clinically superior. With edema produced by egg white in rats as a test system, it was found that the antiphlogistic effect of trypsin could be inhibited by soybean and ovomucoid inhibitor, but only at concentrations 5 to 10 times above those producing inhibition of tryptic activity *in vitro* (TABLE 8). The antiphlogistic action of a number of trypsin derivatives (supplied by F. F. Nord), as measured by minimum effective dosage, bore no relation to their dissociation constants with serum trypsin inhibitor (TABLE 9). It appeared rather to be a function of proteolytic activity. Chronic administration of trypsin to guinea pigs caused only small increases in serum trypsin inhibitor levels (TABLE 10). Results indicate that combination with serum inhibitor does not influence the antiphlogistic action of trypsin.

Identification of the antiphlogistic action of trypsin with permeability modification led us to studies of various chemotherapeutic and pharmacological agents used in conjunction with trypsin. For example it has been

TABLE 8
EFFECT OF SOYBEAN INHIBITOR ON ANTIPHLOGISTIC ACTION OF TRYPSIN

Treatment	Wt. difference (gm.), control leg—edematous leg	Inhibition of edema (%)
Control	1.90 ± .05	—
Trypsin 2 mg./kg.	1.20 ± .15	36
Trypsin 2 mg./kg.	1.18 ± .11	37
Soybean inhibitor 6 mg./kg.	1.82 ± .21	0
Trypsin + soybean inhibitor 6 mg./kg.	1.50 ± .05	21
Trypsin + soybean inhibitor 10 mg./kg.	1.50 ± .10	21
Trypsin + soybean inhibitor 20 mg./kg.	1.65 ± .12	13

TABLE 9

Derivative	K serum inhibitor*	Proteolytic activity*		MED for edema inhibition (mg./kg.)
		Hb	Casein	
Trypsin	2.2×10^{-9}	100	100	2
Acetyl	5.2×10^{-9}	56	75	5
Butyryl	1.1×10^{-7}	22	70	10
Citraconyl	1.5×10^{-7}	17	71	200

* Figures on dissociation constants and proteolytic activity from Bier, Ram, and Nord (1955).

generally observed that only small amounts of some antibiotics can be detected in the spinal fluid following parenteral administration. Studies on the physiological effects of parenterally administered trypsin suggested that this enzyme might increase the permeability of the spinal membranes and thus permit an increased passage of antibiotics from the blood into the spinal fluid. Penicillin was given intramuscularly to trypsin-treated dogs. At intervals small amounts of spinal fluid were removed and assayed for penicillin content by the standard disc-plate method, using *Micrococcus pyogenes* var. *aureus* as the test organism. Results showed a definite increase in penicillin content of the spinal fluid of trypsin-treated dogs over controls, as well as an increase in the time during which an effective level was maintained (FIGURE 2). Thus, in the controls a maximum penicillin level of 0.45 μg./ml. was reached in 2 hours and fell to 0.27 μg./ml. at 3 hours. For the same time periods the values in a set of trypsin-treated animals were 0.81 and 1.06, respectively.

A logical extension of the antibiotic studies was a study of the blood-brain barrier. It appeared that enzymes modifying structures in the capillary wall might affect the passage of substances across the wall into the brain, and that a study of such enzymes might throw some light on the mechanisms of passage across the blood-brain barrier. Hyaluronidase has

TABLE 10
EFFECT OF CHRONIC ADMINISTRATION OF TRYPSIN ON SERUM INHIBITOR LEVELS

Treatment	Percentage substrate destroyed			
	1:80	1:160	1:320	1:640
Control	8.0	11.0	18.0	21.0
Trypsin 1.0 mg./kg. daily	5.6	8.6	15.4	19.2
Trypsin 0.1 mg./kg. daily	6.0	10.0	16.0	20.0

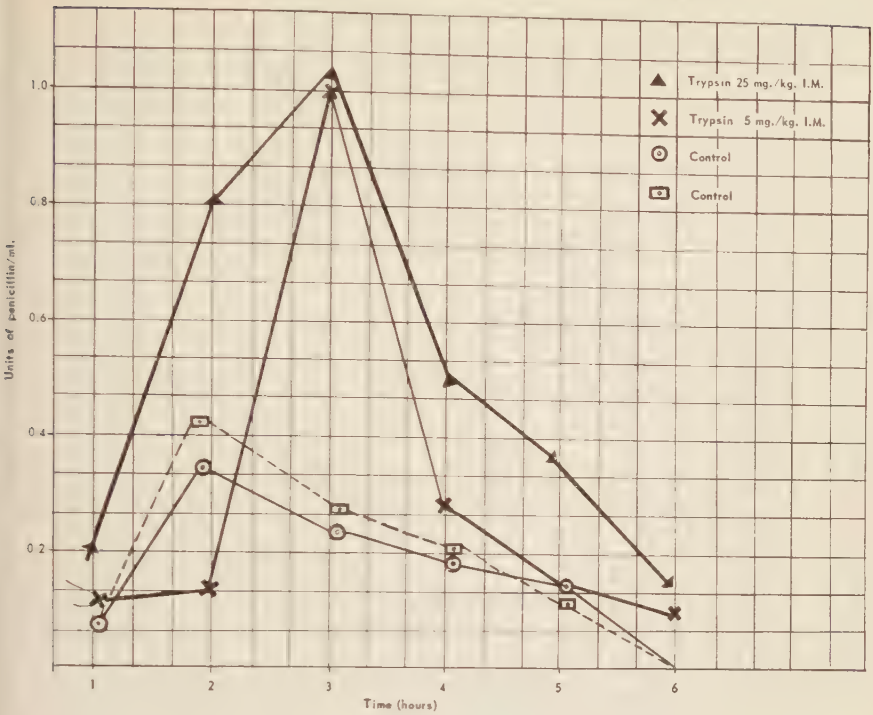

FIGURE 2

been reported (Murakami, 1952) to increase penetration of drugs into the brain. Accordingly, a number of enzymes of varying specificities were investigated. These enzymes were chosen so as to assay the effects of actions on the protein, mucopolysaccharide, lipid, and nucleic acid constituents of the capillary endothelial cell and its supporting structures.

Reduction in the latent period for sodium barbital action was used as a criterion for measuring increases in permeability. The method used was essentially that of Greig and Mayberry (1951). Determinations were done on mice weighing 25 to 30 gm. The substances being tested, in 0.2 ml. of solution, were injected intraperitoneally. After 30 min., sodium barbital, 300 mg./kg. in 0.2 ml. of solution, was injected by tail vein. The latent period was measured by determining the time taken for loss of the righting reflex. Controls receiving barbital alone were run with each experimental group to allow for day-to-day variations in the response of the animals to the barbiturate.

TABLE 11 gives the changes in the period of latency to barbital produced by the various enzymes tested. All figures are averages for groups of 6 animals.

Eserine was tested as a positive control, and the results obtained with this compound confirm those reported by Greig and Mayberry (1951). Of

TABLE 11

CHANGES IN LATENT PERIOD OF BARBITAL PRODUCED BY VARIOUS ENZYMES

Enzyme	Dose (mg./kg.)	Latent period (min.)	P
Control	—	15.0 ± 1.3	
Control	—	15.2 ± 2.1	
Control	—	14.0 ± 1.0	
Control	—	15.3 ± 1.0	
Eserine	0.4	7.2 ± 2.2	<0.01
Trypsin	20	7.2 ± 1.2	<0.01
Trypsin	5	8.7 ± 1.4	<0.01
Trypsin	2	11.7 ± 1.0	0.05
Chymotrypsin	20	6.2 ± 1.2	<0.01
Streptokinase	20	8.1 ± 1.2	<0.01
Plasmin	50	12.0 ± 1.6	>0.05
Lysozyme	50	13.0 ± 3.0	>0.05
Hyaluronidase	4	9.2 ± 1.5	<0.01
Lecithinase	0.1	8.2 ± 1.2	<0.01
Ribonuclease	200	12.8 ± 1.6	>0.05

the enzymes tested, trypsin, chymotrypsin, streptokinase, hyaluronidase, and lecithinase produced significant effects, while plasmin, lysozyme, and ribonuclease did not. It is suggestive that all the enzymes producing positive effects have been shown to be active in inhibiting experimental edemas and to increase tissue permeability as measured by intracutaneous dye spread in rabbits (Beiler and Martin, 1955; Martin *et al.*, 1954; Brendel *et al.*, 1956). Conversely, those enzymes that were inactive in decreasing the latent period to barbital gave negative results in these tests. The minimum effective dose of trypsin in the system reported here is of the same order of magnitude as the dose that will reverse several types of experimental edema in which changes in permeability are involved (Martin *et al.*, 1954; Beiler *et al.*, 1955).

From the results obtained, it would appear that modification of a number of the structures comprising the blood-brain barrier can reduce the latent period of barbital. Thus, from consideration of the enzymes that showed activity, one would assume that effects were obtained by alteration of the protein and mucopolysaccharide constituents of the barrier, and, in the case of lecithinase, by modification of the lipid-water relationships. It is of interest to note that ribonuclease, which would affect a constituent of the cell rather than its supporting structures, was without effect. This is in accord with the concept of Zweifach (1955) that penetration across the capillary wall is largely restricted to its intercellular portion.

Although preliminary experiments with all the enzymes used indicated no hypnotic effects, the possibility remained that some of the positive results obtained might have been due to a potentiation of the action of barbital rather than to an increase in its rate of penetration into the brain. In the case of trypsin, the rate of passage of barbital into the brain was measured directly in order to settle this question. The results are presented in FIGURE

3. Each point on the graph represents an average of 6 experiments. Differences are significant only at the 15-min. interval, where $P = 0.05$.

While these results indicated an increased rate of entrance of barbital into the brain after injection of a standard amount, they gave no information on the effect of changes in blood levels of barbiturate. To investigate this aspect, hexobarbital was used, since its rapid destruction would insure constantly changing blood levels, and its short duration of action would lend itself to convenient measurement. Changes in the duration of its action produced by the various enzymes were measured. Results of this experiment are presented in TABLE 12. The figures again represent the averages for groups of 6 animals. No significant change was observed with any of the substances tested.

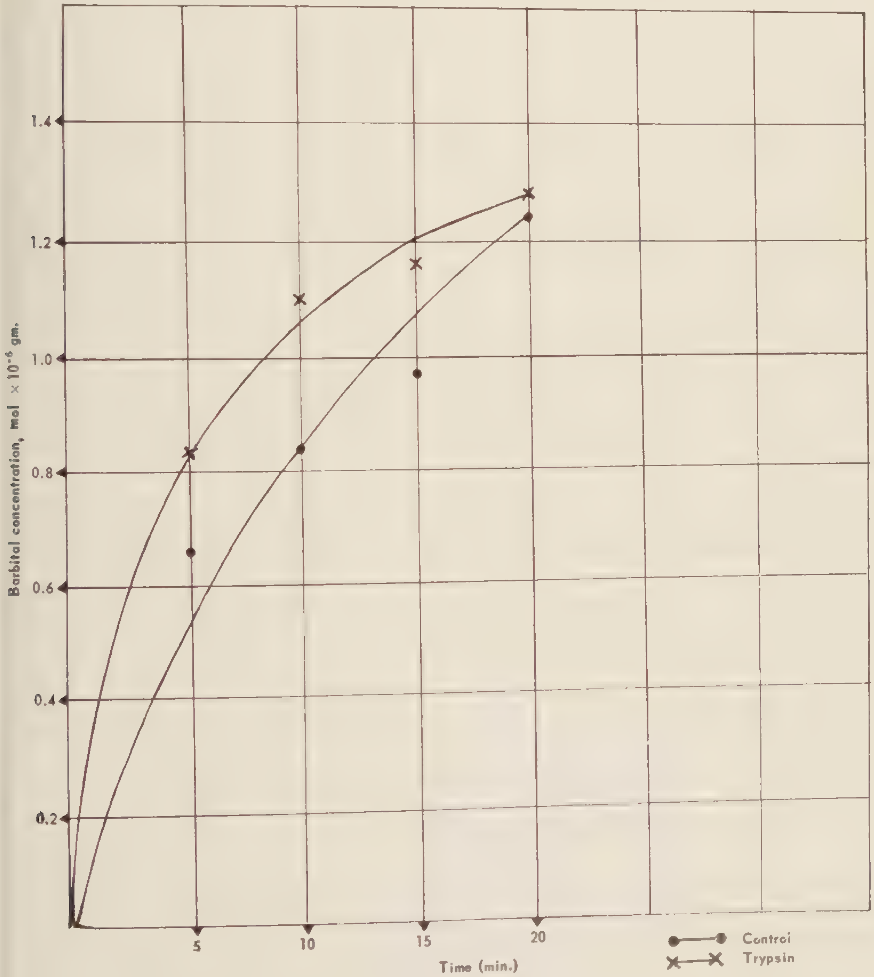

FIGURE 3

TABLE 12

EFFECTS OF VARIOUS ENZYMES ON DURATION OF ACTION OF HEXOBARBITAL

Enzyme	Dose (mg./kg.)	Duration of action (min.)	P
Control	—	29 ± 8	
Control	—	35 ± 6	
Control	—	34 ± 5	
Control	—	34 ± 8	
Control	—	23 ± 3	
Control	—	25 ± 18	
Control	—	30 ± 12	
Trypsin	20	30 ± 6	>0.05
Chymotrypsin	25	25 ± 9	>0.05
Streptokinase	20	29 ± 7	>0.05
Lecithinase	0.1	32 ± 3	>0.05
Hyaluronidase	4.0	34 ± 2	>0.05
Eserine	0.4	44 ± 24	>0.05

With pentobarbital, on the other hand, increases in duration of action were produced by the enzymes used (TABLE 13). The reason for this difference in response between hexobarbital and pentobarbital is not known. It seems possible, however, that differences in the rate of destruction may be involved. Hexobarbital, which has an appreciably shorter period of action than pentobarbital, may be metabolized at a rate rapid enough to counterbalance any effect due to increased brain permeability. It is also possible that, since the rate of entry into the brain is known to vary directly with the rate of destruction of barbiturate (Tatum, 1939), an artificial increase in brain permeability may not produce a more rapid rate of entry than would obtain normally.

The results of this series of experiments indicate that a number of different enzymes can modify the action of barbiturates in the animal body. These enzymes are known to increase vascular permeability, and the findings are

TABLE 13

EFFECT OF PROTEOLYTIC ENZYMES ON DURATION OF ACTION OF PENTOBARBITAL

Enzyme	Dose (mg./kg.)	Duration (min.)	P
Control	—	35 ± 4	
Control	—	26 ± 4	
Control	—	31 ± 7	
Control	—	32 ± 5	
Trypsin	20	60 ± 13	<0.01
Trypsin	10	55 ± 3	<0.01
Trypsin	2	49 ± 11	0.05
Trypsin	1	24 ± 12	>0.05
Chymotrypsin	25	68 ± 10	<0.01
Streptokinase	25	49 ± 14	0.05

consistent with the theory that their effect is due to modification of the permeability of the blood-brain barrier.

Thus, trypsin produces an increase in tissue permeability that is correlated with its antiphlogistic effect (Beiler and Martin, 1955). It is known also to facilitate passage through tissue of normally impenetrant substances (Moss *et al.*, 1955). These actions suggested that, despite its large molecular size, trypsin might cause sufficient increase in permeability in some of the readily accessible mesodermal tissues of the body to facilitate its own absorption. A significant reduction of edema is produced by buccally administered trypsin (TABLE 14). The minimum effective dose, 10 mg./kg., is somewhat higher than that found on subcutaneous injection (Martin *et al.*, 1954). Differences in rates of absorption probably account for this.

Although inhibition of egg-white edema was chosen as the most convenient method for measuring the systemic effects of trypsin, such effects can be demonstrated by other methods after buccal administration of the enzyme. For example, the area of spread of intracutaneously injected India ink in rabbits was found to be increased 100 per cent over control values in animals given trypsin buccally; this same effect was obtained on parenteral administration of the enzyme (Beiler and Martin, 1955). As might be expected, absorption is not confined to the buccal membrane. Significant inhibition of egg-white edema was obtained when trypsin was given intravaginally in a carbowax-based tablet; there is, therefore, absorption from this site.

The recent report of Bastian *et al.* (1956) recommended determination of plasma trypsin or chymotrypsin by means of the esterase activity of these enzymes. Concentrations of the order of 10 μg. per ml. were determinable. Following intravenous administration, both factors appeared in demonstrable quantities in the plasma but, following intramuscular administration in dosages as high as 7.5 mg./kg., no enzymatic activity could be detected. These workers concluded that "the anti-inflammatory effect noted following intramuscular injection of these enzymes is probably not mediated by their systemic absorption." This conclusion does not seem warranted, for many reasons. First, in man the maximum dosage of trypsin given is 5 mg. to a 70-kg. man. On calculation, this gives a maximal possible blood level of 0.6 μg./ml., which is far below the limit of the esterase method. Second,

TABLE 14

INHIBITION OF EGG-WHITE EDEMA IN RATS BY BUCCALLY ADMINISTERED TRYPSIN

Dose trypsin (mg./kg.)	Weight difference (gm.), edematous leg—control leg	Inhibition of edema (%)
Control	1.94 ± .11	—
20	0.80 ± .12	55
10	1.46 ± .08	25
5	1.82 ± .21	0
2	2.02 ± .06	0
Control	1.80 ± .16	0

a dosage of 7.5 mg./kg., administered intramuscularly, would give a calculated level of about 75 μg. per ml. if it were all absorbed instantaneously. Bastian and his associates took samples of plasma at 30 min. and at 1 hour, whereas we know from our own observations that the maximal absorption of trypsin in such dosage from an intramuscular site occurs in approximately 24 hours. In other words, the blood level of trypsin is maximal after 24 hours if one assumes that this level is correlated with maximal antiedema effect. If 50 per cent of the possible plasma trypsin is blocked by inhibitors and if we assume only a 24-hour period of absorption, the concentration in the blood at any given time would be about 1.6 μg. per ml. Again, we find a value well below the limit of the method.

Since there is a lower limit on the amount of enzyme detectable by the esterase method and an upper limit on the amount of enzyme that can be administered intramuscularly without causing tissue damage, it seems improbable that the problem can be solved by such techniques.

If we assume blood levels of trypsin in the range of 0.5 μg. per ml., then direct action of trypsin could be related to the power of fibrin to concentrate the enzyme or to a large difference in the effective concentrations for proteolytic and esterase activity and the effective concentration for depolymerase action. Either or both of these proposals could be correct.

While the evidence presented does not establish the concept of direct action of trypsin, it does tend to support this view. As yet no alternative proposal fits the experimental observations. The concept, then, fits existing knowledge, but existing knowledge is not sufficiently extensive to warrant conclusion. There is little question that modifications of permeability underlie the observed phenomena; adequate experimental data are available to substantiate this conclusion.

In summary, therefore, it can be stated that a direct action of trypsin is probable and that trypsin-induced modifications of permeability correlate with existing data.

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Discussion of the Paper

H. Neurath (*Department of Biochemistry, University of Washington, Seattle, Wash.*): In regard to your experiments with trypsin at 37°, *p*H 7.5: in the absence of calcium and of specific substrates trypsin deteriorates rapidly, at the rate of 50 per cent in the first hour. I should like to ask what effect this factor has on studies designed to establish the minimum dose.

G. J. Martin: We have no direct evidence with respect to that. We have recently been doing a great deal of work on the stabilization of trypsin by combination with a substrate. I think that *in vivo* administration would parallel that kind of experiment. If you provide trypsin with a substrate you can keep it completely stable for long periods; in some of our experiments it remained stable up to a year. I would not think that the decomposition of trypsin would be a significant factor in *in vivo* experiments.

L. S. Kryle (*Department of Therapeutics, New York University College of Medicine, New York, N. Y.*): I wonder if Martin could explain the effect of trypsin in human disease by the same theory that he now advances in his experimental work, especially in the light of the fact that the dosage used is far below his minimal figure of 2 mg./kg.

G. J. Martin: I think that the answer to this requires a complete knowledge of inhibitory factors, or antitrypsin concentrations, even at a local inflammatory site. Resolution of the problem may come only when we achieve good tagged trypsin. This we are trying to do. At the present time I do not believe that any of the classical techniques for determining tryptic activity are adequate to answer the problem. I feel, however, that there is no basic discrepancy inherent in dosage differentials. The question must be related to the medium in which action occurs, and to antifactors and rates of destruction.

J. M. Miller (*Surgical Service, Veterans Administration Hospital, Fort Howard, Md.*): I am intrigued by the concept of increasing permeability of the blood-brain barrier by the use of trypsin. Clinically we have observed that in inflammation the passage of penicillin to the spinal fluid is enhanced by inflammation: ordinarily one does not get good concentrations of penicillin in the spinal fluid in normal individuals; they are increased when inflammation is present. I wonder if you would explain that seeming paradox.

G. J. Martin: I would think that this would be related to the time factor. In inflammation, the pore size first increases, and this is followed by blockage later on. Thus, in the initial phase of an inflammatory reaction there will be increased permeability and one would expect more antibiotic to pass through.

Question: What sort of an inflammatory response does egg-white edema represent? Is there such a tremendous outpouring of inflammatory cells that proteolytic enzymes derived from leukocytes might be present? Is this a true inflammatory response?

G. J. Martin: Whether or not this system is related to clinical inflammatory states is open to debate, and whether any presently utilized technique can be said fully and wholly to correlate with the clinical condition is questionable. This is why most laboratories use a number of different experimental inflammations in testing, as we have done.

Question: Have you studied the inhibitor level in the fluid in the inflamed area? We do know that a tremendous amount of inhibitor comes out in serum in any inflammatory fluid space.

G. J. Martin: We have studied the trypsin inhibitor levels only systemically in blood, not locally. It is simply a matter of methodology. We have not been able to work out a technique to obtain adequate fluid by a controllable and simple method. However, if you found any rather marked modification locally, I would not think that it would be related to a systemic effect. We obtained no significant modification systemically.

THE EFFECT OF STREPTOCOCCAL ENZYMES AND OTHER AGENTS ON EXPERIMENTAL INFLAMMATION

By Samuel Gordon and Frank B. Ablondi

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The role of proteolytic enzymes in the inflammatory process has been considered by many investigators. Menkin's studies[1] on the cellular response in inflammation have shown that tissue breakdown products produce inflammatory reactions. Menkin has suggested that some of the characteristics of inflammation for example, emigration of leukocytes from blood vessels, their accumulation at a site of injury, and increased capillary permeability are due to a substance that is polypeptide in nature. He also showed[2] that prolonged incubation of serum or of crystalline serum albumin with trypsin yielded products that increased capillary permeability and caused emigration of leukocytes. Duthie and Chain[3] have demonstrated that substances with similar inflammation-inducing properties could be obtained by digesting different proteins with proteolytic enzymes. These substances were obtained from serum albumin, egg albumin, casein, fibrin, serum globulin, gelatin, and edestin. These authors concluded that the active principle was a polypeptide, and made attempts to purify it. Cullumbine and Rydon[4] also investigated protein digests and came to the same conclusion. By careful analysis of the polypeptides, Spector[5] was able to establish that, in the case of a peptic digest of fibrin, a number of peptides of average chain length of from 8 to 14 amino acid residues could cause leukocyte emigration and increased capillary permeability.

The evidence presented above suggested that polypeptide or other protein breakdown materials were responsible, at least in part, for the events occurring during the inflammatory process. If such were the case, it seemed conceivable that modification of the proteolytic status of the organism might influence the course of inflammation. Indeed, the work of Innerfield[6] in the study of the clinical effects of trypsin seems to lend support to this hypothesis.

In 1933 Tillett[7] showed that hemolytic streptococci were capable of lysing fibrin clots. Later Christensen[8] and Kaplan[9] demonstrated that these streptococcal preparations contained a kinase that was capable of converting the inactive plasminogen of certain species into the active proteolytic enzyme, plasmin. This enzyme, in addition to its ability to attack fibrin, also digested casein and gelatin[10] and certain amino acid esters.[11] Since a streptokinase (SK) preparation was readily available to us in the form of Varidase*, we thought it would be of interest to investigate the effect of this preparation on experimental inflammation.

Our first consideration was the selection of a method of inducing inflammation. Examination of the literature revealed a perplexing number of methods that have been advocated as a means of evaluating the anti-inflammatory effect of various substances. These methods range from administration of such simple irritants as egg white, dextran, or yeast extract, into the rat[12-14] to formation of granulomas by means of cotton pellets[15] or croton oil in an air pouch.[16] Since it had been shown that SK is capable of activating the plasminogen of the rabbit[17] but not that of the rat,[18] the rabbit was selected as the laboratory animal of choice.

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* Trademark of the American Cyanamid Co. for a mixture of streptococcal enzymes consisting of streptokinase, streptodornase, other proteins, and phosphate buffer. Lederle Laboratories Division, Pearl River, N. Y.

Preliminary experiments involving subcutaneous administration in the rabbit of irritants such as formaldehyde, croton oil, mustard oil, or diphtheria toxin proved this approach unsuitable in our laboratory for precise experimental study. Encouraging results were obtained in the study of the inflammation produced by the instillation of mustard oil into the rabbit eye.[19] The following experimental procedure was adopted:

Twelve male New Zealand albino rabbits, approximately 2 kg. in weight, were used in each test. The animals were kept at least a day in the laboratory to check their condition and allow them to adjust to laboratory environment. In random order 6 animals were given the material to be tested, and 6 controls received saline (solutions administered intravenously in 1 ml. doses). Approximately 15 min. after the administration of the test substance or saline, 0.3 ml. of a 15 per cent mustard oil solution (redistilled artificial mustard oil* in mineral oil) was instilled under the lid of one eye. Approximately 2 hours later the extent of inflammation was noted by scoring from 1 to 4 depending upon the degree of inflammation. A score of 4 (FIGURE 1a) was characterized by marked edema of the conjunctiva, causing the palpebral margins to bulge. This score was given to an eye in which there was involvement of both the upper and lower lids and of the nictitating membrane. There was marked redness and secretion of fluid, and the eyelids were almost completely closed. A score of 1 (FIGURE 1b) consisted of greatly reduced edema. However, some indication of irritation of the conjunctiva was still present. The eyelids were open almost to normal position. Scores between 1½ and 3½ represented intermediate conditions. The animals were graded by several observers and the results were evaluated by the rank method of Wilcoxon.[20] The total of the individual scores was used to rank the animals. The sum of the ranks of the treated rabbits was calculated and the probability that the group treatments differed was determined from the tables of Wilcoxon.[21]

TABLE 1 shows data of an experiment in which 20,000 units of SK in the form of Varidase were administered intravenously. It will be noted that the animals that received SK displayed lower ranks than the controls. In this experiment the value of the rank sum for the SK-treated animals did not exceed 23, thus representing a probability of 1 in 100 or less that the results could have arisen by chance.

Inasmuch as we were interested primarily in the effect of different streptococcal preparations on experimental inflammation, we undertook to test these preparations. Various concentrations of SK in the form of Varidase were tested. Each dose was made up in 1 ml. of 0.9 per cent saline and was

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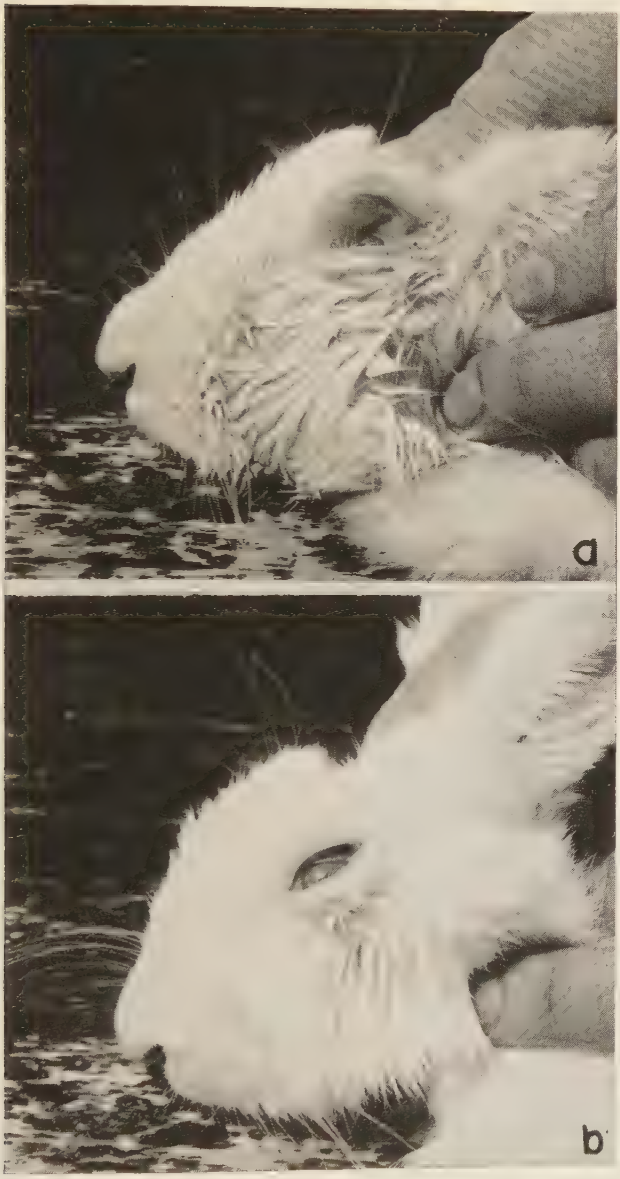
* Amend Drug & Chemical Co., Inc., New York, N. Y.



FIGURES 1a and 1b. (a) Example of score "4" inflammation; note marked edema of the conjunctiva exposed by separating the lids. (b) Example of score "1" inflammation; note reduced edema.

TABLE 1
EFFECT OF 20,000 UNITS SK (VARIDASE) ON EXPERIMENTAL EDEMA

Rabbit No.	Treatment	Scores at 2 hours by 3 observers 1	2	3	Totals	Rank
6	S	1½	1½	1½	4½	6
2	SK	1	1	1	3	2.5
4	S	4	4	4	12	11
1	SK	1½	2	1½	5	7
5	S	2½	2	2½	7	8
7	SK	1	1	1	3	2.5
3	S	4	4	4	12	11
10	SK	1	1	1	3	2.5
8	S	4	4	3½	11½	9
11	SK	1½	1½	1	4	5
9	S	4	4	4	12	11
12	SK	1	1	1	3	2.5

Sum	P
27	0.05
24	0.02
23	0.01

From Wilcoxon[21]

The sum of the ranks of the SK-treated animals is 22.0 ($P < 0.01$).

TABLE 2
EFFECT OF VARIOUS DOSES OF VARIDASE ON EXPERIMENTAL EDEMA

Dose units SK × 10^3	Sum of ranks of treated animals	P
50	21	0.01
20*	27.5 22 26	0.01
10*	22.5 21 36.5	0.01
5*	35 26.5	>0.05
1	41	>0.05

* More than one experiment was performed and P calculated on the basis of combined results.

administered intravenously. TABLE 2 shows the data obtained. From the column of probabilities it is evident that a significant difference exists between Varidase-treated animals and controls, even at concentrations as low as 10,000 units of SK. It should be emphasized that the determination of the minimum effective dose by this method has its limitations.

TABLE 3
EFFECT OF PURIFIED SD (FREE OF SK) AND PURIFIED SK (FREE OF SD) ON EXPERIMENTAL EDEMA

Preparation	Sum of ranks of treated animals	P
Purified SD	44 42.5	>0.05
Purified SK	22.5 31	0.01

Since Varidase is a mixture of streptococcal proteins consisting in part of SK and streptodornase (SD), the effect of a purified SD preparation was studied. This preparation displayed negligible SK activity and contained approximately the same concentration of SD as is present in 100,000 units of Varidase. TABLE 3 shows the results of this study. It will be noted that there was no significant difference between the controls and the SD-treated animals. Furthermore, partially purified preparations of SK, obtained by chromatography and displaying negligible SD activity, were capable of preventing the inflammatory changes produced by mustard oil. The data indicate that SD is not the factor responsible for inhibiting the edema.

In order to ascertain whether a correlation exists between the antiedema effect of an SK preparation and its ability to induce a demonstrable fibrinolytic response, the presence of a fibrinolytic system was determined. This was accomplished by withdrawing a small sample of blood into a capillary tube and noting the lysis time after coagulation. TABLE 4 shows the fibrinolytic response obtained with various doses of SK (as Varidase). These results are the averages of several determinations and indicate that a demonstrable fibrinolytic response is observed at a level of 500 units of SK. All

TABLE 4
FIBRINOLYTIC RESPONSE (AVERAGE LYSIS TIME IN MINUTES) OBSERVED WITH VARIOUS DOSES OF VARIDASE

Dose units SK × 10³	Time interval between SK administration and withdrawal of blood sample			
	1 min.	15 min.	30 min.	60 min.
1000	15	7.5	13	17
100	7	5	6.5	25
10	9	16	33	—
0.5	26	—	—	—

(—) No lysis occurred during the 6 hours of observation.

TABLE 5
AVERAGE LYSIS TIME OF BLOOD SAMPLE REMOVED AFTER ADMINISTRATION OF 10,000 UNITS SK (VARIDASE)

	Time interval between SK administration and withdrawal of blood samples		
	1 min.	15 min.	30 min.
*Cortisone treated	18.2 (14–20)		
*Control	8.4 (7–10)	20.4 (14–34)	36.4 (23–59)

* 5 Animals in each group.
(—) No lysis occurred during the 8 hours of observation.

effective antiedema concentrations of SK produced a demonstrable fibrinolytic response in the rabbit.

If fibrin deposition is involved in the edema of this type of inflammation, the prevention of fibrin formation by a suitable agent should result in suppression of edema. Accordingly, 10,000 units of heparin, a dose sufficient to render the blood incoagulable during the experimental period, was tested. It was found to be ineffective in reducing the edema, thus suggesting that fibrin deposition is not a major factor in the induction of edema in this type of inflammation. Whether circulating proteolytic activity is a necessary prerequisite for the antiedema effect of streptococcal preparations cannot be answered at this time. The results with heparin in which prevention of fibrin deposition did not alter the course of the inflammation points out the complexity of this problem. In addition, the intravenous administration of sodium salicylate at a level of 300 mg./kg. was found to inhibit the edema effectively without evoking a detectable fibrinolytic response. Salicylates have been reported previously to inhibit this type of experimental edema.[19, 23]

Varidase preparations have been shown to contain a hyaluronidase.[22] Since streptococcal hyaluronidase free of SK is unavailable, testicular hyaluronidase was studied. A dose of fifteen hundred USP units of testicular hyaluronidase* was administered intravenously and found to be inactive.

It was of interest to determine the effect of cortisone on this experimental syndrome. Rabbits were given daily doses of 20 mg. of cortisone acetate and 40,000 units of penicillin intramuscularly for a period of 7 days. The animals were then challenged with mustard oil and scored 2 hours later as described previously. No significant difference was found between the cortisone-treated and the control animals. No detectable fibrinolytic activity was observed in blood samples taken from the animals.

It is of further interest to note that in other experiments with cortisone we have found that treated rabbits displayed a diminished fibrinolytic response to SK as compared to untreated animals. TABLE 5 shows a comparison between the fibrinolytic responses of treated and control animals after the

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* Cudahy Laboratories, Omaha, Neb.

Table 6
Effect of Various Agents on Mustard-Oil Edema of the Rabbit Eye

Agent	Antiedema activity	Fibrinolytic activity*
Varidase	+	+
SK (purified)	+	+
SD (purified)	−	−
Testicular hyaluronidase	−	−
Heparin	−	not determined
Cortisone acetate	−	−
Sodium salicylate	+	−

* Determined in a blood sample removed approximately 5 minutes after administration of each agent.

intravenous administration of 10,000 units SK. The treated animals received 20 mg. cortisone acetate and 40,000 units of penicillin daily for 6 days.

The diminished response to SK in the cortisone-treated animals may possibly be explained on the basis of reports in the literature. For example, Moll[24] recently reported that administration of cortisone to rabbits caused an increase in the serum trypsin inhibitors. In addition, Kwaan[25] found an inhibition of clot lysis in man after administration of corticotrophin.

In table 6 are tabulated the results obtained with the various agents studied.

Our experiments may be summarized as follows: inflammation was induced by instillation of mustard oil into the eye of the rabbit. The intravenous administration of certain streptococcal preparations prior to the induction of inflammation was shown to be effective in decreasing the severity of the inflammation so produced. The activity of these preparations cannot be explained by the presence of SD, which, in purified form and free of SK, was found to be inactive. Furthermore, a partially purified preparation of SK, free of SD, was active in decreasing the edema. Sodium salicylate also served as an effective antiedema agent without producing fibrinolysis. Although detectable fibrinolytic activity was present after the administration of every effective level of SK, the need for an active circulating fibrinolytic system in order to effect a reduction of this type of experimental edema has not been established. Pretreatment with testicular hyaluronidase, heparin, or cortisone acetate was ineffective.

Acknowledgment

The authors gratefully acknowledge the capable participation of W. P. Cekleniak and P. K. Siiteri in these experiments. They are also indebted to E. C. De Renzo for his advice and for help in the preparation of the manuscript.

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Discussion of the Paper

P. S. Norman (*Johns Hopkins Medical School, Baltimore, Md.*): You have made the point that cortisone reduces the fibrinolytic response to streptokinase, but does not change the inflammation produced by your test. Does pretreatment with cortisone change the response to streptokinase when you try to inhibit the edema?

S. Gordon: Unfortunately, we have not tried this.

L. S. Kryle (*Department of Therapeutics, New York University College of Medicine, New York, N. Y.*): Did you have occasion to use chymotrypsin or trypsin in similar experiments?

S. Gordon: Yes, we have. With trypsin we have been unable to give sufficiently large doses intravenously. At the dose levels that we have used, approximately 5 mg. of trypsin per rabbit intravenously, we have not achieved the reversal of the edema as established by our criterion. However, at much larger doses, for example, 100 mg. of trypsin intramuscularly, we do see an effect on the edema. This might be explained by the fact that trypsin is readily inactivated, making it necessary to have high levels.

CLINICAL AND EXPERIMENTAL STUDIES ON FIBRINOLYTIC ENZYMES*

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and George L. Collins

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In vitro fibrinolytic activity has been reported for many proteolytic enzymes of animal and plant origin. A fibrinolytic factor was described in blood as early as 1893 (Dastre, 1893). For several years our group has been engaged in developing quantitative methods for the evaluation of potential fibrinolytic agents *in vivo*. Pharmacological properties, toxicology, and histopathological effects of these agents were studied. Promising preparations were selected for clinical trial in cancer patients with thromboembolic complications. On the basis of clinical experience, attempts are being made to produce improved preparations and to work out optimal dose schedules. Although this work is still in progress, it may be appropriate to review our completed experiments and to present a preliminary report on current studies.

Methods and Materials

In Vivo *Testing of Fibrinolytic Agents*

Development of a quantitative method using I^{131}-labeled fibrinogen was described previously (Ambrus, Back, Mihalyi, and Ambrus, 1956). Fibrinogen was labeled according to the method of Mihalyi and Laki (1952).

Dogs were anesthetized with 25 mg./kg. sodium pentobarbital I.V. supplemented with additional doses when needed. Femoral and jugular vein clots were produced as follows:

A section of the vein to be thrombosed was isolated between two artery clamps. A fine polyethylene catheter was inserted and ligated into one of the side branches of the segment, connected to a blunt needle and a syringe containing the fibrinogen solution (FIGURES 1 and 2). Other side branches were ligated. Blood from the segment was withdrawn into the syringe, where it mixed with 0.1 ml. of I^{131}-labeled fibrinogen solution prepared as described previously (Ambrus, Back, Mihalyi, and Ambrus, 1956). The mixture was reinjected and withdrawn three times to ensure thorough mixing. After the last injection, the syringe was exchanged for another one containing 50 units of thrombin (Parke, Davis & Company) in 0.05 ml. physiological saline; the content of the segment was withdrawn into this syringe and rapidly reinjected. The side branch was ligated and the polyethylene tubing removed. A semiconstricting aluminum wire ligature was placed close to

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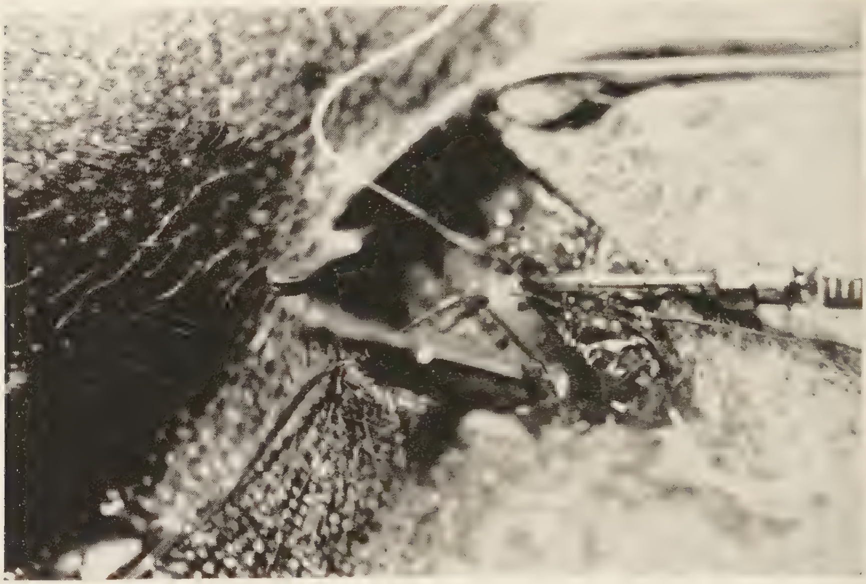

FIGURE 1. Femoral vein with side branch cannulated and connected to tuberculin syringe for injection of I^{131}-labeled fibrinogen.

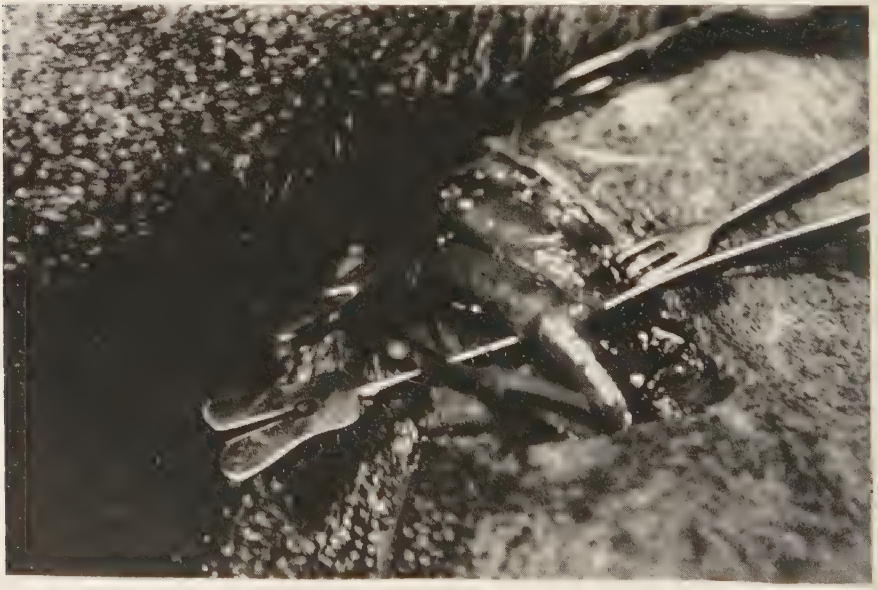

FIGURE 2. Thrombosed vessel with semiconstricting ligature.

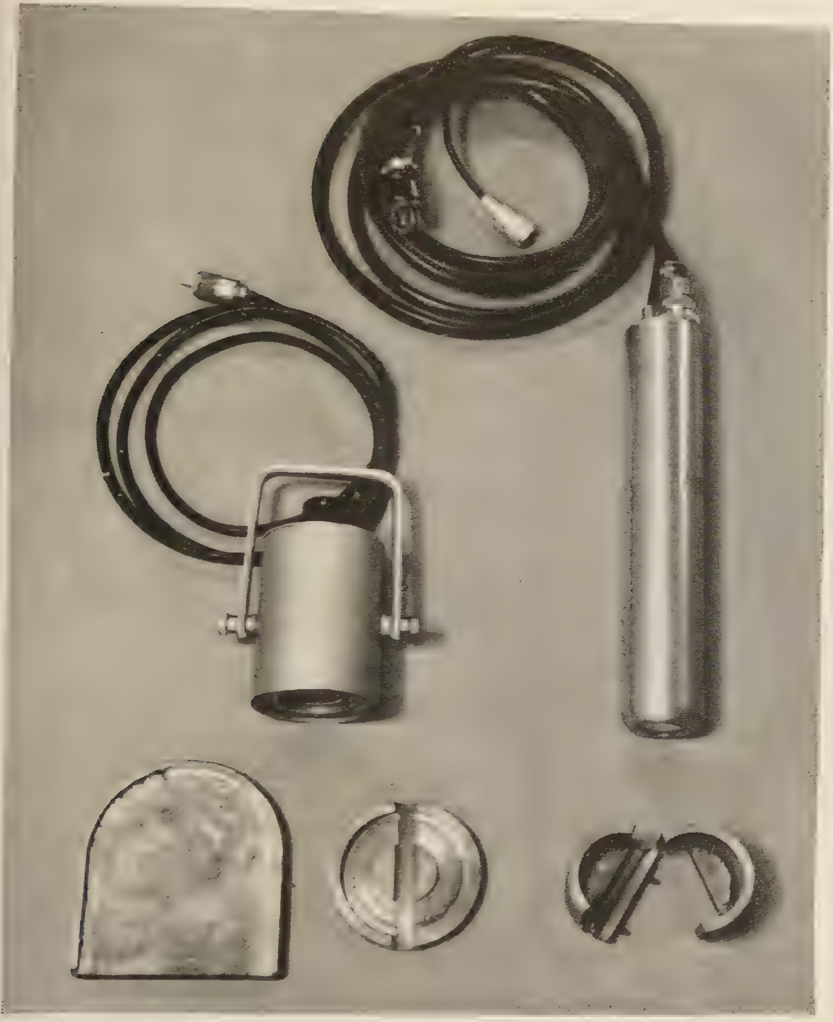

FIGURE 3. Left to right. Upper row: (1) Geiger-Mueller tube used in preliminary experiments; (2) scintillation counter. Lower row: (1) lead shield for rabbit ears; (2) shield for isolated vessels built to accept Geiger-Mueller tube; (3) shield to accept scintillation counter; this can be assembled around the thrombosed vessel.

the proximal artery clamp and adjusted to prevent the thrombus from passing the ligature in case the clot were to become loose, yet to allow flow through the constricted area if it were to become lysed. The placement of this ligature appeared to be critical. In preliminary experiments, it was observed that without this ligature clots might slip away and might be undetectable by routine autopsy. Using radioactive fibrin, however, such broken-up clots could always be detected by autopsy under the guidance of a scintillation counter and by histological and radioautographic examination of

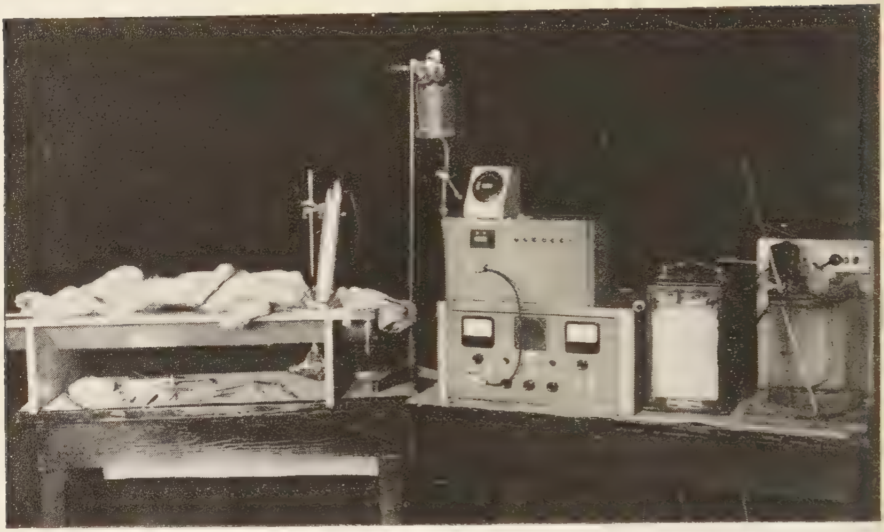

FIGURE 4. Experimental arrangement for the continuous recording of radioactivity from I^{131}-labeled clots *in situ*. Lead shield shown enclosed around thrombosed segment of the jugular vein. The scintillation counter is inserted into the shield and connected to the radiation rate meter. The rate meter is connected to the Esterline recorder and to an apparatus to record total counts (on top of the rate meter). The constant temperature bath beside the Esterline recorder is used for rapid determination of clotting factors. The instrument used for transillumination of thrombosed vessels is seen under the dog board.

active areas. It is believed that many positive results in the literature may be due to the slipping away of nonradioactive clots. Half an hour after completing the above procedure, the artery clamps were removed. A specially built lead shield (FIGURE 3) was assembled around the thrombosed vessel, so that the latter rested in the groove of the shield. A scintillation counter was inserted into the shield and was connected to a radiation rate meter, which in turn was connected to an Esterline recorder. FIGURE 4 shows the entire experimental arrangement. FIGURE 5 shows segments of a typical Esterline record. These values represent, of course, uncorrected count rates. In calculating results, correction was made for decay of the isotope, dead time of the tube, and background radiation; no correction was necessary for changes in calibration of the instrument or for backscatter. Radioactivity of the clots was usually followed continuously during infusion of the agents under test and for 4 hours thereafter; then tetracycline powder was applied to the area and the skin was closed with wound clips. About 24 hours after beginning treatment the wound was opened, local radioactivity determined, and the next treatment started. Before each operation 1 million units of penicillin and 250 mg. of streptomycin were given subcutaneously.

Femoral artery clots were produced in the same manner as venous clots, except that semiconstricting ligatures were placed distal to the clot. Procedures employed in monkeys were similar to those used in dogs. In unanesthetized rabbits, marginal ear vein clots were produced as follows:

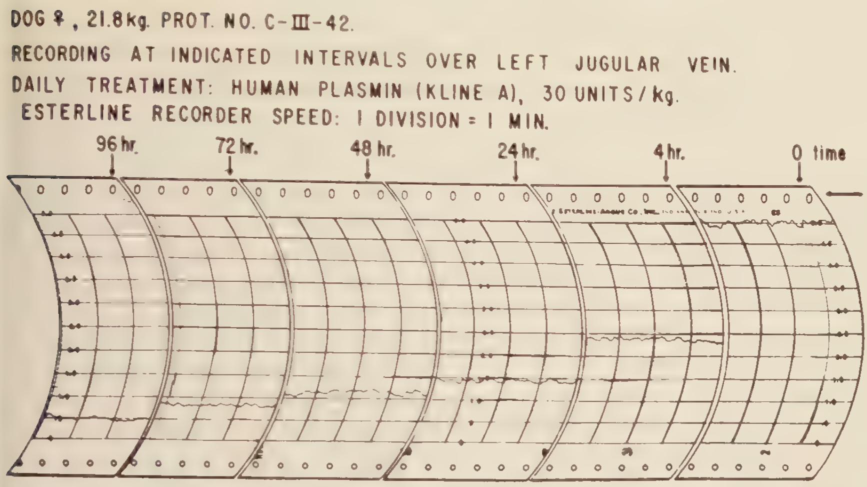

FIGURE 5. Segments from a typical Esterline record of radioactivity of clot during daily treatment with plasmin.

A segment of the vein was isolated between two artery clamps. All side branches were compressed with long curved hemostats. A 27-gauge needle was inserted into the segment, its content withdrawn, and a mixture of 0.05 ml. I^{131}-labeled fibrinogen and 0.05 ml. 5 per cent sodium morrhuate was injected. Half an hour later the distal artery clamp and the hemostats were removed. After an additional half hour the proximal artery clamp was also removed. Semiconstricting ligatures could not be used in these experiments. Slipping away of clots occurred occasionally.

Criteria of Evaluation of Lysis of Peripheral Thrombi

(1) *Radioactivity of the clot.* Radioactivity was recorded during and after treatment. After appropriate corrections (see above), these values were remarkably constant during a five-day period in untreated animals. FIGURE 6 shows values of a femoral artery clot in a control dog; FIGURE 7 those of a marginal ear vein clot in a control rabbit. In animals in which lysis of the clot occurred, slight radioactivity could be detected in the liver and high activity in the urine; no activity was found in the thyroid or in the salivary glands. Apparently the effective fibrinolytic enzymes used in this study do not liberate free I^{131}. Probably low-molecular-weight I^{131}-labeled peptides are produced and are removed chiefly through the kidneys.

(2) *Phlebography or arteriography.* Diodrast was injected proximal to arterial clots and distal to venous clots through polyethylene catheters introduced through side branches of the thrombosed vessel or directly into the vessels through 30-gauge needles. Flow of the contrast medium through the thrombosed area was observed by fluoroscopy. Results were expressed in arbitrary units as follows: 0 = not passable, 1 = partially passable, 2 = fully passable. FIGURE 8 shows a phlebogram of a normal rabbit ear; FIGURE 9 that of a thrombosed marginal ear vein (passability: 0). In

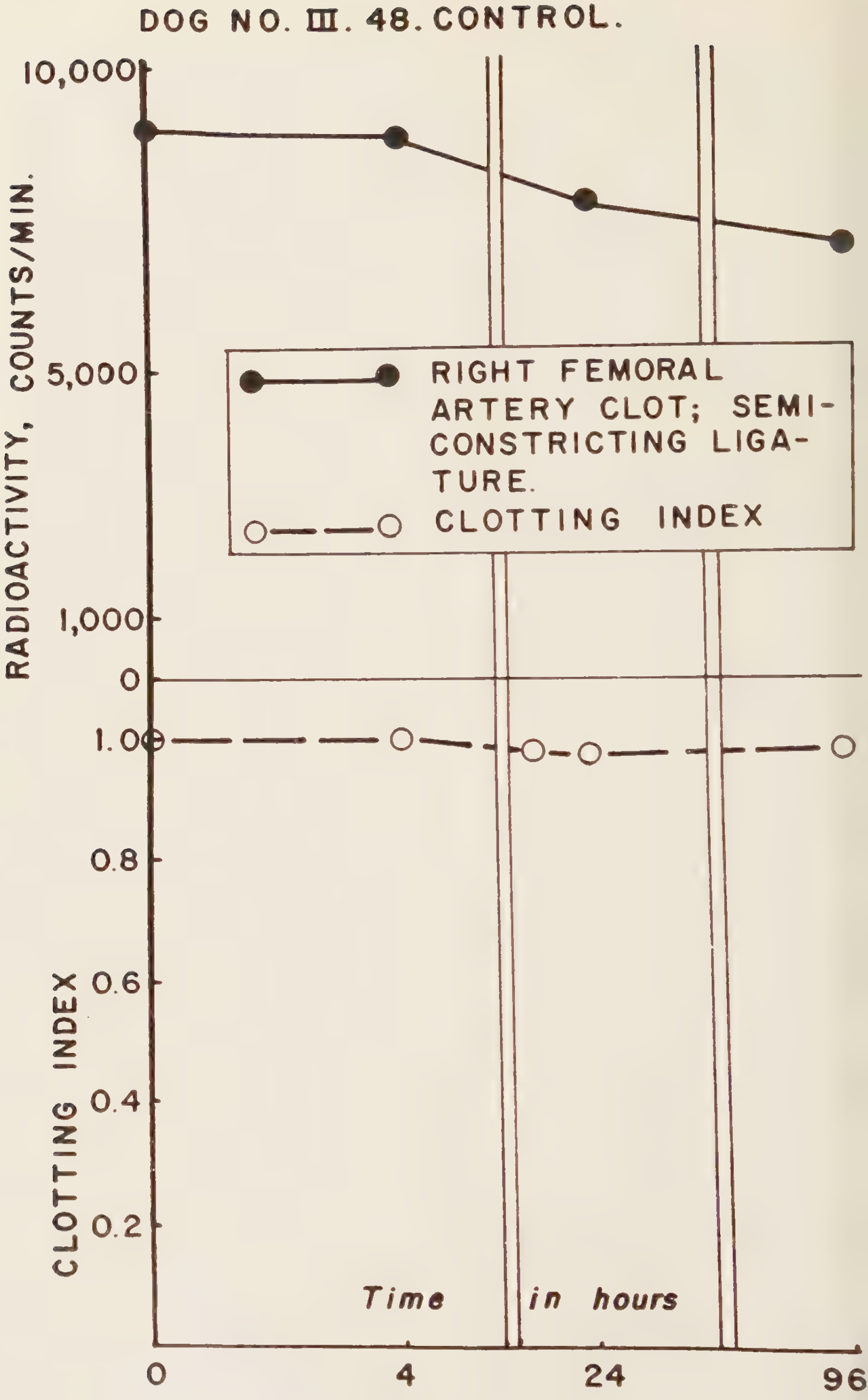

FIGURE 6. Radioactivity of I^{131}-labeled femoral artery clot in a control dog.

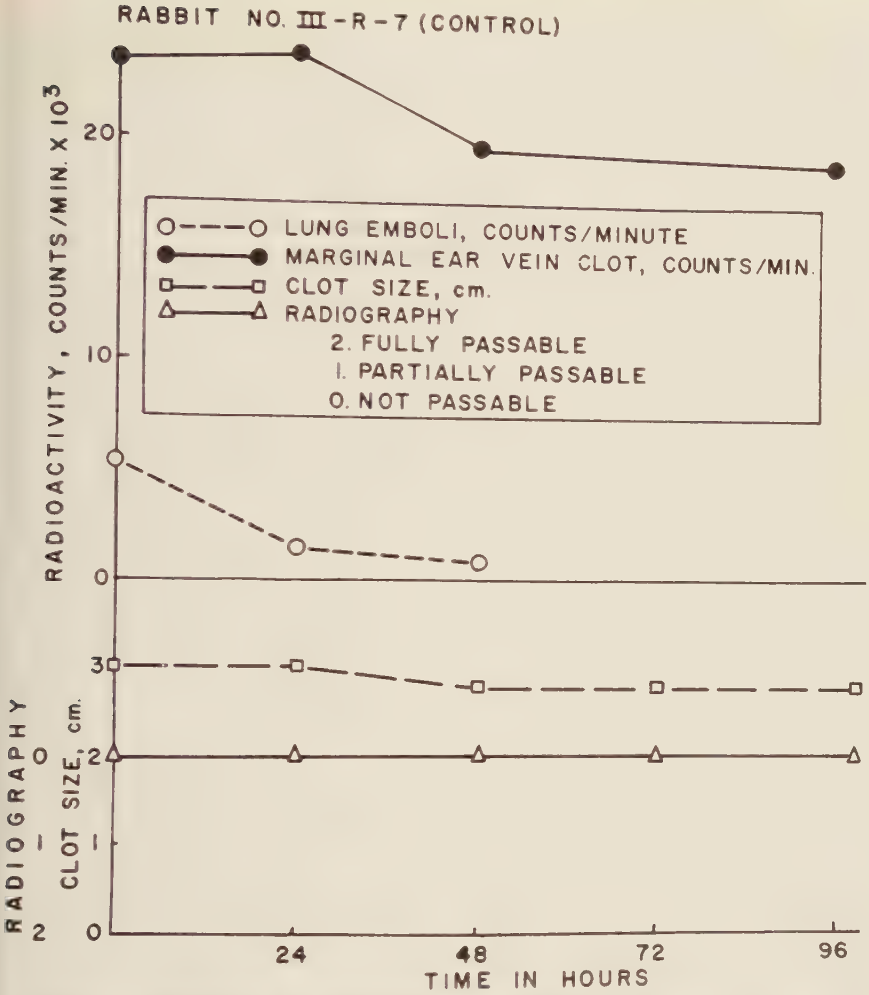

FIGURE 7. Radioactivity and radiographic and transillumination data of I^{131}-labeled marginal ear vein clot and pulmonary emboli in a control rabbit.

FIGURES 7 and 17 are graphic representations of phlebographic data. FIGURE 10 shows a phlebogram of the femoral vein of a dog with a fresh clot and a semiconstricting ligature (passability: 1). FIGURE 11 shows a thrombosed femoral vein of a dog with a completely constricting proximal ligature. It can be seen that the X-ray contrast medium does impregnate the meshwork of the clot. In fresh clots there is some flow through the thrombosed area (FIGURE 10). In veins with clots and completely constricting ligatures, there is significant backflow from the clot; the contrast medium is removed through collaterals. After completing the injections of Diodrast, radio-opacity disappeared from the clots within thirty minutes. These findings

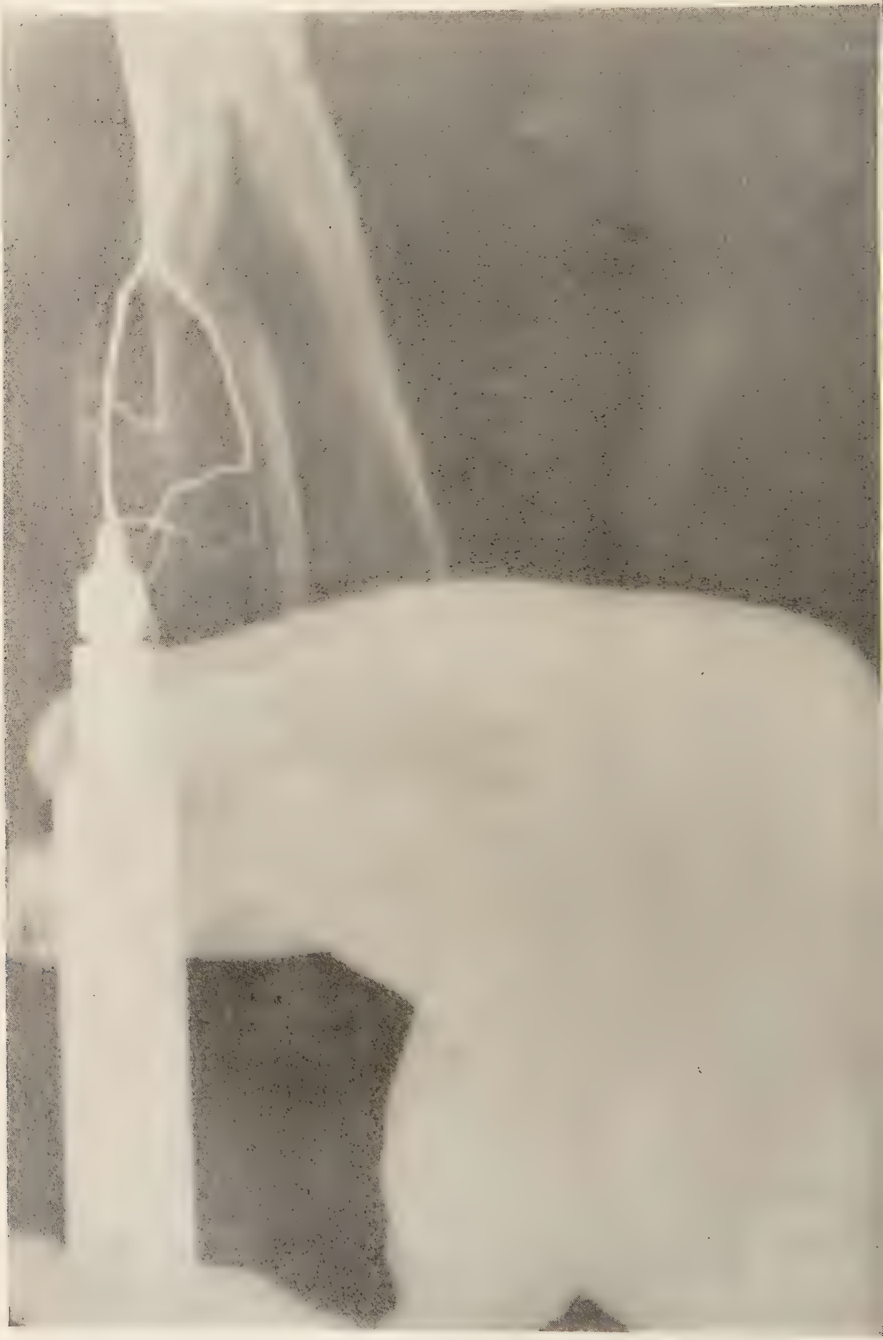

FIGURE 8. Phlebogram of a normal rabbit ear injected with Diodrast.

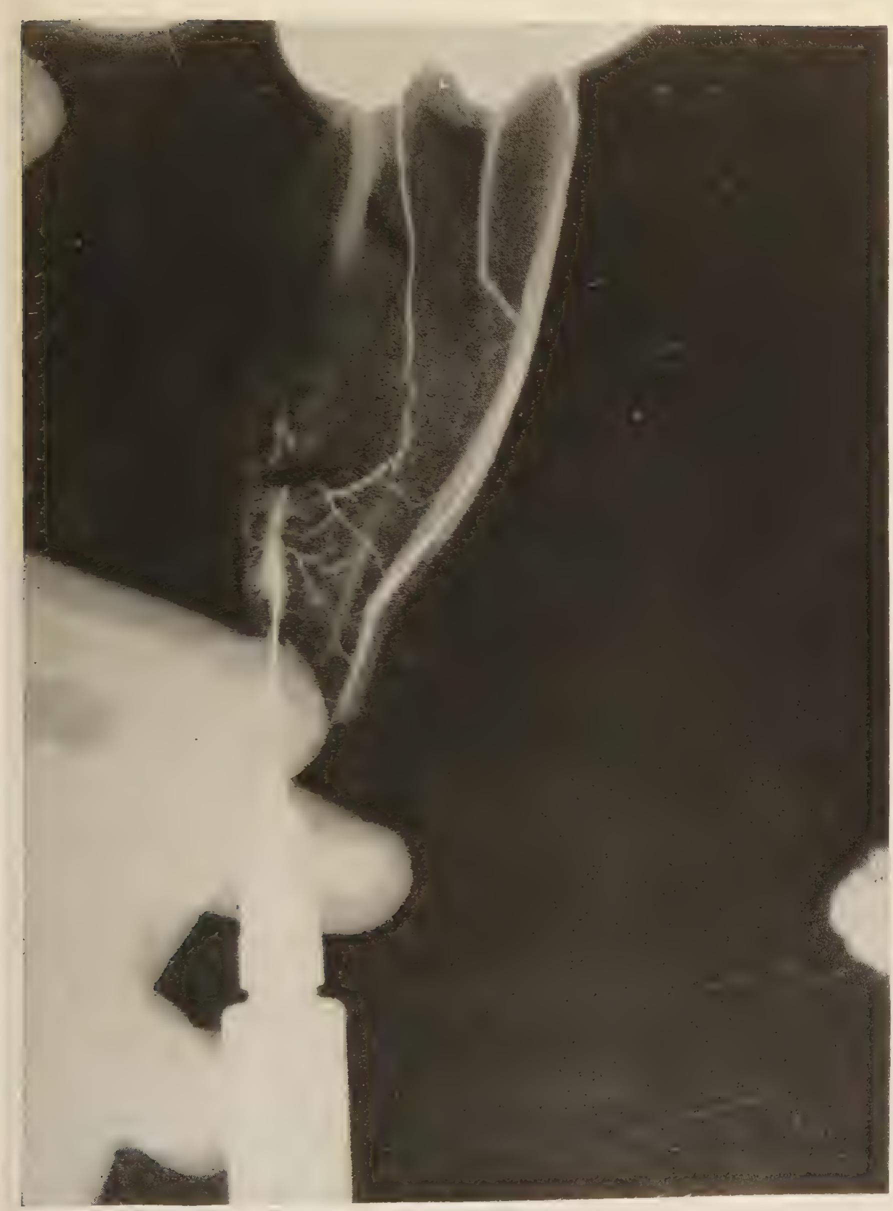

FIGURE 9. Phlebogram of a rabbit ear with a thrombosed marginal ear vein.

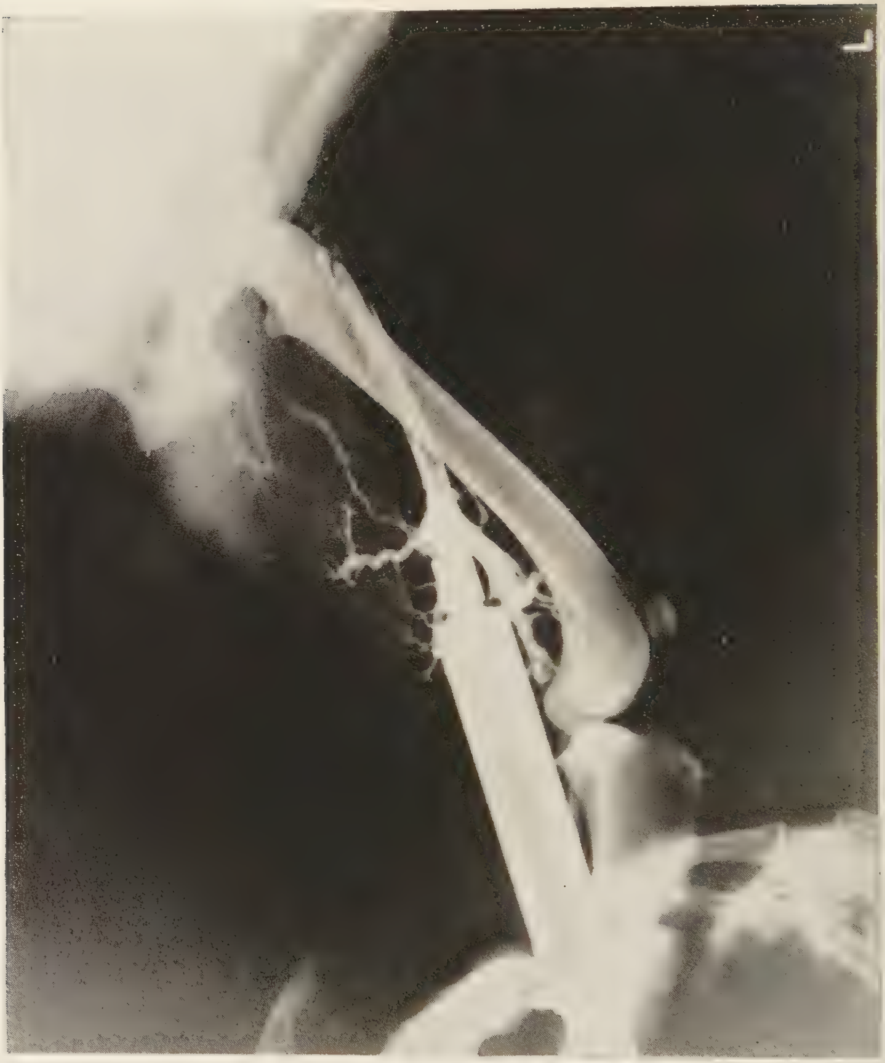

FIGURE 10. Phlebogram of the femoral vein of a dog with a fresh clot and a semiconstricting ligature. Note the flow of contrast medium through the clot.

suggest that fibrinolytic agents injected intravenously will reach the fibrin fibers of clots even when the latter completely obstruct flow in certain vessels.

(3) *Transillumination.* FIGURE 12 shows a rabbit ear with a marginal vein clot transilluminated with a powerful light source. It appeared that the length of the clot could be estimated. Femoral or jugular vein clots were transilluminated with a bent-tube transilluminator (FIGURE 4). Estimates of clot length, particularly with the latter type of clot, proved to be quite inaccurate and were used only as an additional aid in evaluating results. In

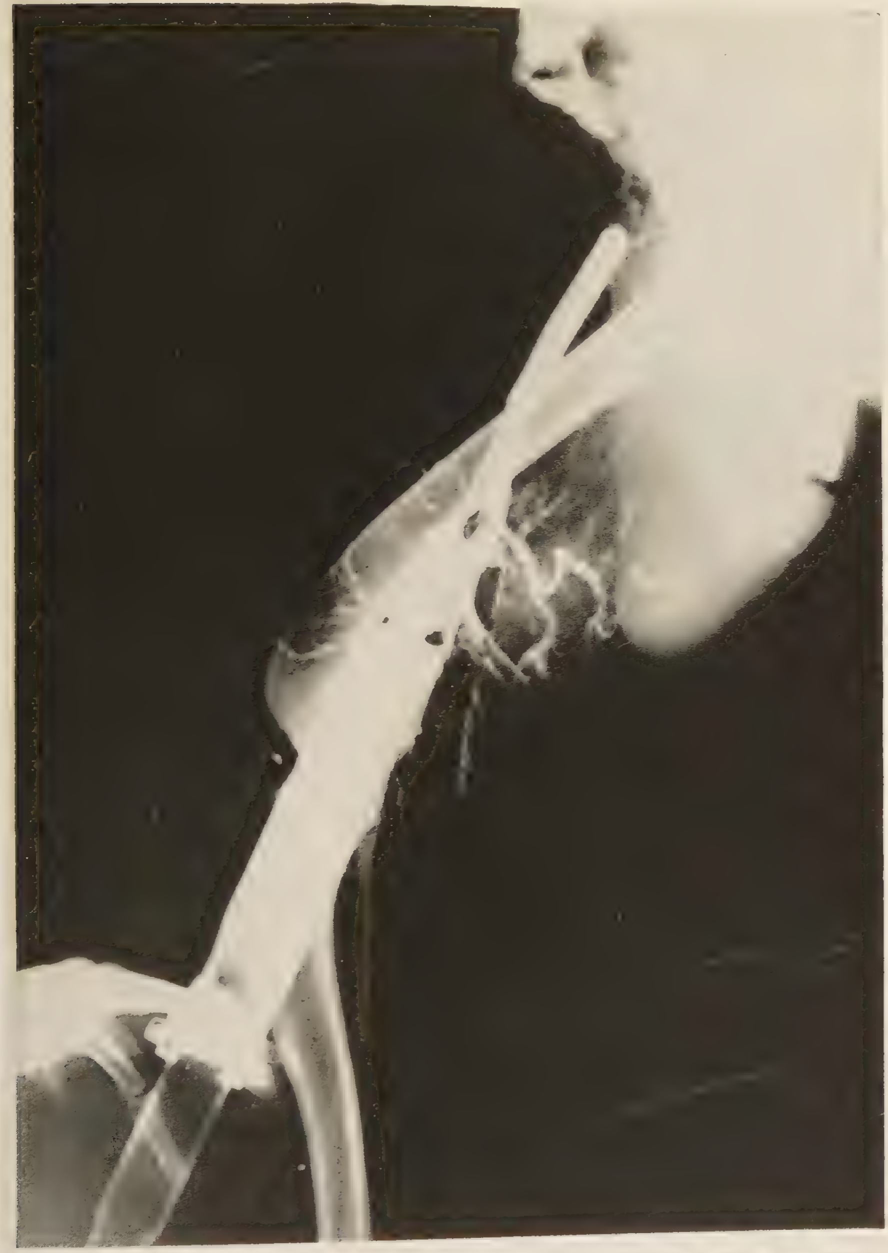

FIGURE 11. Phlebogram of a thrombosed femoral vein of a dog with a completely constricting proximal ligature. Note the backflow of contrast medium through the collaterals.

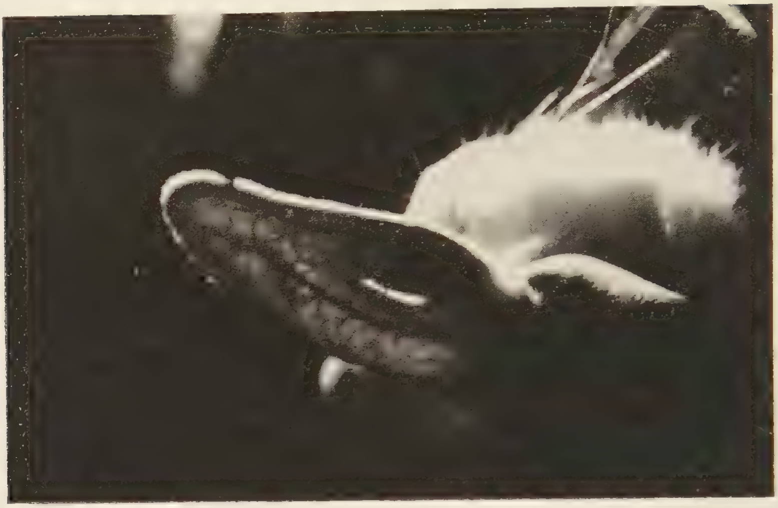

FIGURE 12. Transillumination of rabbit ear with marginal vein clot.

rabbit ear clots the results were expressed in cm. of clot length. In femoral artery and vein clots and jugular vein clots of dogs the following arbitrary units were used: 3, full clot; 2, clot with some fluid phase; 1, decreased clot size with more fluid phase; 0, no clot.

(4) *Autopsy and histopathological examination.* Histopathological examination was undertaken on all clots after terminating the experiment.

Pulmonary and Peripheral Emboli

Pulmonary and peripheral emboli were produced as follows: five milliliters of venous blood was withdrawn and mixed with 2 ml. of I^{131}-labeled fibrinogen and 50 units of thrombin. The resulting clot was homogenized in a Potter-Elvehjem-type homogenizer, the supernate was decanted, and the fibrin suspended in 5 ml. physiological saline. One half to two milliliters was injected into an ear vein, in rabbits, to produce pulmonary emboli, or in dogs, through a catheterized side branch into the femoral artery to produce peripheral emboli. The embolized areas were scanned with a scintillation counter, and areas of equal activity were marked on the shaved skin with fuchsin or picric acid. In untreated animals, pulmonary emboli exhibited a relatively rapid decline of radioactivity (FIGURE 7), while peripheral emboli seemed to disappear more slowly. Because of changes in geometry of counting, these methods were considered qualitative or, at best, semiquantitative.

Methods to Differentiate Between Anticoagulant and Fibrinolytic Effects in Vivo

Most of the fibrinolytic agents used prolonged the clotting time and affected a number of coagulation factors. The following method was devised to differentiate between the relative significance of fibrinolytic and anticoagulant effects. Labeled clots were produced in jugular veins of dogs as described before, except that an additional maxillary branch was prepared and

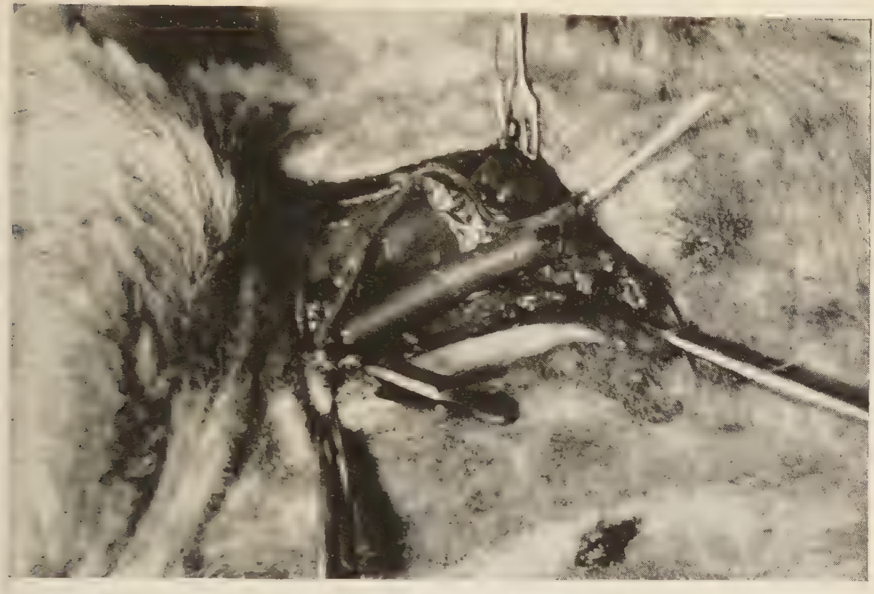

FIGURE 13. Jugular vein of dog with polyethylene rod inserted for formation of canalized clot.

a polyethylene rod inserted through it into the area to be thrombosed (FIGURE 13). At the end of the procedure, this rod was removed, the branch was ligated, and serial phlebograms were taken. FIGURE 14 shows flow through a normal jugular vein before thrombosis; FIGURE 15 depicts flow through a canalized clot, and FIGURE 16 flow through the same clot one hour later. It can be seen that the central canal becomes rapidly obliterated by new clot formation. Thus, clots consisting of a cortex of I^{131}-labeled fibrinogen and a core of nonradioactive material were produced. Decrease of radioactivity indicated lysis of the original clot, and delay of obstruction of the central canal indicated *in vivo* anticoagulant effect. It will be seen that most proteolytic enzymes tested were effective anticoagulants in high doses, but had little fibrinolytic effect.

Analysis of Members of the Fibrinolysin System

(1) *Plasmin activity.* This activity was determined by a modification of the method of Loomis, George, and Ryder (1947). One Loomis unit of plasmin was defined as the amount capable of dissolving 0.6 ml. of a 0.3 per cent bovine fibrin clot at 45° C. in 2 minutes in an imidazole buffer of *p*H 7.2. Bovine fibrinogen (Armour) was purified 3 times by the method of Laki (1951). From the plasma, or solutions of plasmin concentrates to be analyzed, 0.2 ml. was pipetted into a 10 × 75 mm. test tube in a 45° C. water bath. To this was added 20 units of bovine thrombin (Parke, Davis & Company) in 0.1 ml. of imidazole buffer of *p*H 7.2. Into this mixture 0.3 ml. of a 0.6 per cent purified bovine fibrinogen solution was pipetted, and a stop watch was

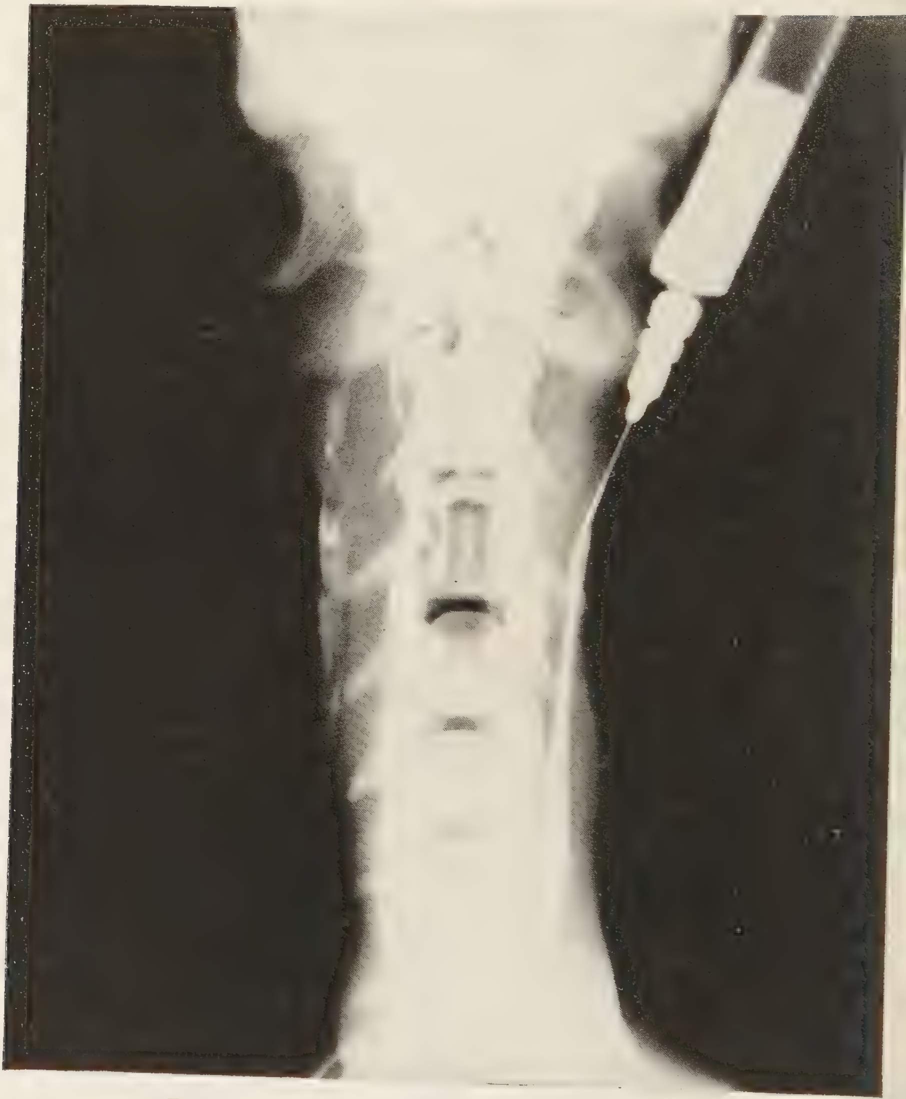

FIGURE 14. Phlebogram of normal jugular vein of dog.

started. As soon as a clot formed, a capillary tube was inserted into the middle of the clot so that the tip of the capillary was slightly above the bottom of the tube. It was found important to select tubes of uniform dimensions. The tip of the tube was broken to produce an irregular edge in order to decrease interference by surface-tension phenomena. The end point was taken as the time when the fluid in the capillary rose to the level of the clot. This end point usually occurred a few seconds before complete lysis of the clot, but it was found to be more accurate and reproducible than

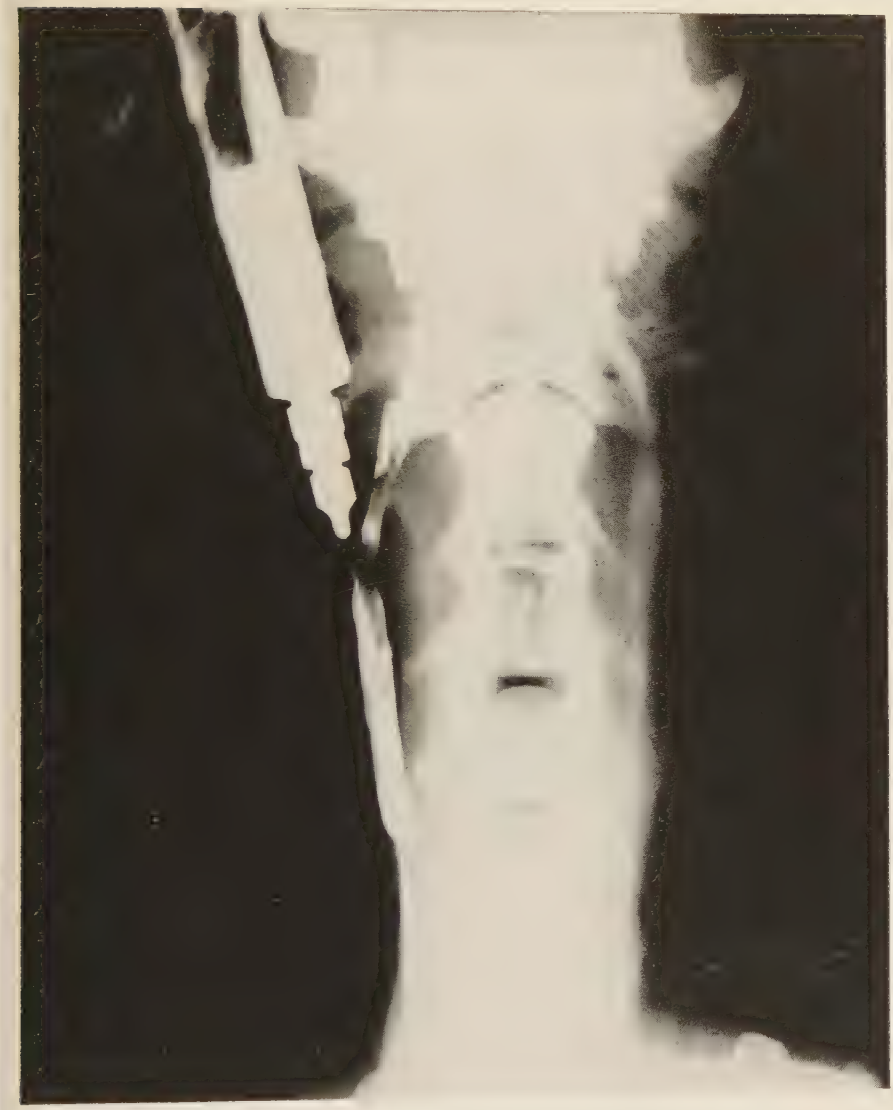

FIGURE 15. Phlebogram of jugular vein after formation of canalized clot. Note flow in central canal.

measurements based on complete lysis. All determinations were done in duplicate. Standard curves were constructed by plotting known amounts of purified plasmin preparations against lysis times. Taking the quantity producing lysis in 2 minutes as 1 unit, lysis times were plotted against units. Lysis times of samples tested were converted to units with the aid of these curves.

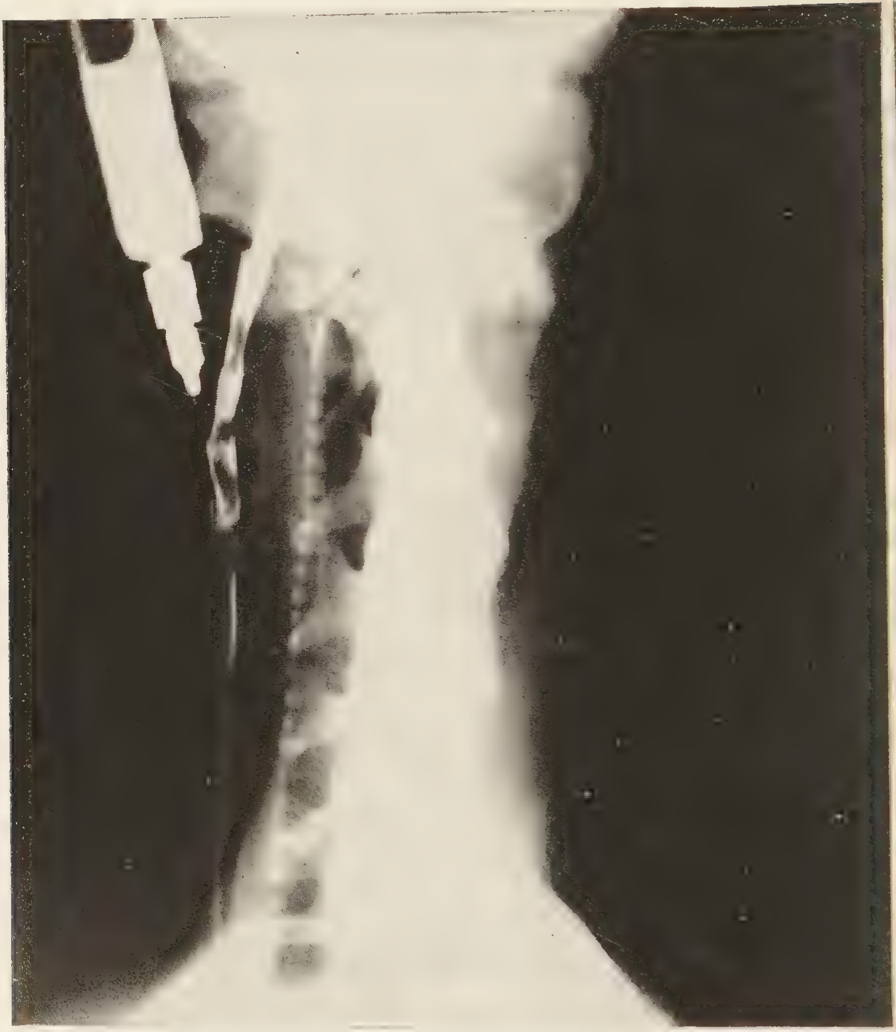

FIGURE 16. Phlebogram of jugular vein shown in FIGURE 15 after one hour. Note obstruction of flow in central canal.

(2) *Plasminogen levels.* Human plasminogen levels were determined as follows: 0.3 ml. of the serum to be analyzed was mixed with 3000 Christensen units of streptokinase (Varidase), dissolved in 0.3 ml. of imidazole buffer and incubated for 20 minutes at 28° C. Of this mixture, 0.2 ml. was tested for plasmin activity as described above. One unit of plasminogen was defined as the amount that can be converted by streptokinase (SK) into 1 unit of plasmin. If free plasmin was found in the sample, the units of plasmin were deducted from the total to obtain the units of plasminogen.

(3) *Activator activity.* Bovine plasminogen was prepared by the method

of Loomis, George, and Ryder (1947). Three units of bovine plasminogen was dissolved in 0.3 ml. of imidazole buffer; 0.3 ml. of the serum or the plasmin solution to be tested was added, and the mixture was incubated at 28° C. for 20 minutes. From this mixture 0.2 ml. was tested for plasmin activity as described above. One unit of activator was defined as the activity capable of producing 1 unit of bovine plasmin. Correction was made for plasmin activity. Bovine plasminogen was used in this assay since, in contrast to human plasminogen, it is not activated by streptokinase alone; bovine plasminogen activator is produced from the interaction of streptokinase and human plasminogen.

(4) *Antiplasmin levels.* One unit of antiplasmin was defined as the amount capable of neutralizing 1 unit of plasmin after incubation for 60 minutes at 28° C. It was found necessary to use plasmin preparations without activator activity. If this precaution was not observed, the test measured the result of two opposed processes occurring simultaneously: (1) the conversion of plasminogen in the sample to plasmin by the activator; and (2) neutralization of plasmin by antiplasmin. Human plasmin free from activator activity was prepared by the method of Kline (alcohol- and acid-purified plasmin as described under "enzyme preparations." Six units of such purified human plasmin in 0.5 ml. imidazole buffer was mixed with 0.1 ml. of the serum to be tested and the mixture was incubated for 60 minutes at 28° C. From this mixture 0.2 ml. was tested for remaining plasmin activity, as described above.

(5) *Antistreptokinase.* A dilution series was made from the serum to be tested, and 0.4 ml. of each dilution was incubated with 100 units SK at 37° C. for about 1 hour. Of each of these mixtures 0.2 ml. was added to 2 units of human plasminogen (Kline, fraction A), incubated for 20 minutes at 28° C., and tested for plasmin activity. The highest serum dilution that was capable of preventing the formation of plasmin (by neutralizing SK) was designated antistreptokinase titer. This test is considered to be semiquantitative.

Sources of error. The fibrinogen used in the above procedures was found to be essentially free from contamination with plasmin and plasminogen. On the other hand, the bovine thrombin used contained traces of plasminogen. This represents a source of error in the testing of some plasmin preparations. If these preparations possess activator activity, the plasminogen contamination of bovine thrombin will be converted to plasmin and will increase the values obtained. In the testing of blood samples from treated patients or animals this factor was not involved, since none of the samples discussed exhibited activator activity. Further details of procedures for the estimation of members of the fibrinolysin system and methods for the elimination of sources of error will be reported elsewhere.

Biochemical and Pharmacological Methods

Routine biochemical determinations, pharmacological and toxicological tests were performed using accepted procedures, as in previous publications (Ambrus, Back, Mihalyi, and Ambrus, 1956). In the figures "clotting index" is given instead of prothrombin time; this is defined as the ratio of prothrombin activity to the value before treatment.

Fibrinogen levels were determined by Fisher's (1955) modification of the methods of Ratnoff (1951) and Andersch and Gibson (1933, 1944). Into each of a duplicate set of round-bottom 50 ml. polyethylene tubes containing 0.5 ml. clean, crushed Pyrex glass the following solutions were pipetted in this order: 8.9 ml. 0.9 per cent NaCl, 0.1 ml. saline containing 5 units of thrombin, and 1.0 ml. of the plasma to be tested. After standing for 15 minutes in an ice bath, the tubes were centrifuged in a refrigerated centrifuge, the supernate was decanted, and the clot was washed 3 times with 25 ml. cold saline. Strong biuret reagent was prepared according to the method of Andersch and Gibson (1933, 1944); 5 ml. of this reagent and 10 ml. of saline were added to the clot and agitated until the clot dissolved. Optical density of the resulting blue solution was read at 550 mμ against a freshly prepared blank of 4 ml. saline and 2 ml. strong biuret reagent. The results were read from a calibration curve.

Clinical Studies

All patients in this series suffered from incurable neoplastic disorders and thromboembolic complications. Blood samples were taken before, during, and at various time intervals after treatment. The Lee-White coagulation time was performed at the bedside as a guide to dosage. Measurement of prothrombin time and a fibrinogen screening test were undertaken within a few minutes after obtaining a blood sample. The latter test was performed by adding to 0.1 ml. plasma, 5 units of thrombin in 0.1 ml. saline. Plasma containing 100 mg. per cent fibrinogen or more will clot within 30 seconds. Important prolongation of coagulation time or prothrombin time, or decrease of fibrinogen level was considered as an indication to discontinue therapy or to administer whole blood and or human fibrinogen after completion of treatment. In addition, the following were determined: red- and white-cell count; thrombocyte count; differential count; hemoglobin; hematocrit; fibrinogen level; clot retraction; spontaneous lysis of clot at 28° C.; and lysis at 28° C. of 0.2 ml. samples of dilution series of plasma, each clotted with 5 units thrombin. Measurements were also made of the following members of the fibrinolysin system: plasmin, plasminogen, antiplasmin, activator activity, and antistreptokinase. Occasionally Ac-globulin, proconvertin, and convertin were also determined.

The following clinical measurements were taken to record side effects: oral, rectal, and skin temperatures; BMR; ECG; blood pressure; and respiratory rate. Criteria for therapeutic results were as follows. In arterial occlusion: (1) clinical signs (color demarcation line, pulse in the thrombosed limbs); (2) skin temperature; (3) skin oxygen tension, recorded by the polarographic method of Montgomery and Horwitz (1948), as modified by Urbach, Noel, and Gordy (1955), using the platinum electrode (oxygen cathode) described by Davies and Brink (1942). In venous occlusion: (1) clinical signs (color, subjective); (2) measurement of the circumference of the affected extremities at points marked with a dermograph; (3) cuff test of Lowenberg (1954), based on the observation that if blood-pressure cuffs are placed on extremities with thrombophlebitis, pain is experienced by the patient at lower inflation pressures than in normal limbs.

Enzymes

The following enzyme preparations were tested: (1) crude lyophilized pancreatic protease (Worthington Laboratories, Freehold, N. J.); (2) trypsin, recrystallized from Tryptar (The Armour Laboratories, Kankakee, Ill.); (3) chymotrypsin, recrystallized (Armour Laboratories); (4) carboxypeptidase prepared by the method of Anson (1937) and crystallized three times (Worthington Laboratories); (5) papain (Merck & Co., Inc., Rahway, N. J.); (6) ficin (Mann Research Laboratories, Inc., New York, N. Y.); (7) Varidase (streptokinase-streptodornase, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.); (8) bovine plasmin activated with chloroform, prepared by the method of Loomis, George, and Ryder (1947) (Parke, Davis & Company, Detroit, Mich.). Human plasmin preparations: (9) acid extract of Cohn's fraction III activated with streptokinase (SK), prepared by W. Baumgarten (Merck Sharp & Dohme, Division of Merck & Co., Inc., Philadelphia, Pa.); (10) human plasminogen prepared by the method of Fletcher (1954) and activated with SK; (11) human plasminogen prepared by the method of Kline (1954) and activated with SK; (12) preparation 11 further purified with alcohol fractionation by the method of Fishman and Kline (1956) to remove free SK (kindly supplied by D. Kline); (13) preparation 12, in which SK was inactivated by acid treatment, by one of the following procedures: (a) the preparation was adjusted to *p*H 2, then to *p*H 11, and finally to *p*H 7.2 (see Kline's paper in this monograph); (b) adjusted to *p*H 2, then to *p*H 7.2; or (c) on the basis of the studies of Troll and Sherry (1955), the preparation was brought to *p*H 2, 1 *M* NaCl added, centrifuged, and the precipitate redissolved at *p*H 7.2; and (14) spontaneously activated human plasminogen prepared by the method of Alkjaersig and Sherry (1956). Human lung mitochondria were prepared from 0.25 *M* sucrose homogenates by the method of Schneider and Hogeboom (1950).

Solvents and Stability

Plasmin preparations 12 and 13 were dissolved in 5 per cent dextrose in water, all others in physiological saline. With all of the physiological solvents tested preparation 8 (bovine plasmin) contained visible particles. This preparation was therefore suspended in saline, centrifuged, and the supernate filtered through Mandler filters Nos. 8 and 16. All preparations were dissolved immediately before use. Plasmin preparations were found to decrease in activity when exposed in dilute solutions to room temperature for one or more hours. Infusion bottles were placed into neoprene jackets containing dry ice.

RESULTS

Comparison of in Vivo *Fibrinolytic Activity of Proteolytic Enzymes*

I^{131}-Labeled arterial and venous clots and pulmonary and peripheral emboli were produced in dogs and in rabbits. The therapeutic effects of a number of proteolytic enzymes were studied in the maximal tolerated doses. More promising preparations were also given daily for five days in the maximal chronically tolerated doses. In active preparations the minimal effective dose was established, and a detailed study was undertaken of their pharmacological and biochemical properties. Enzymes considered for clinical trial were tested for activity and toxicity in monkeys, and procedures were worked out for the preparation of sterile products.

Table 1 shows the maximal acute and chronic tolerated doses in dogs and in rabbits of the enzymes used. Table 2 summarizes the experimental results. Detailed reports of the fibrinolytic and pharmacological activities of some of these enzymes have been published previously (Ambrus, Ambrus, Back, Goldstein, and Harrisson, 1956; Ambrus, Back, Mihalyi, and Ambrus, 1956; Back, Ambrus, Goldstein, and Harrisson, 1956; Ambrus, Back, Goldstein, Ambrus, and Harrisson, 1957).

Crude pancreatic protease, trypsin, chymotrypsin, papain, and ficin exhibited no fibrinolytic activity in maximal acutely or chronically tolerated doses. These doses proved to be effective *in vivo* anticoagulants in the canalized-clot test. The fibrinogen level was significantly lowered and the prothrombin time lengthened. In certain instances the blood became incoagulable, and histopathological study revealed extensive hemorrhages. Hypotension was observed upon rapid intravenous infusion of the maximal

Table 1
Maximal Tolerated Doses (per Kilogram)

Enzymes	Dog Slow I.V. infusion		Rabbit I.V. injection	
	Acute	Chronic Daily dose for 1 week	Acute	Chronic Daily dose for 1 week
Crude pancreatic protease	25 mg.			
Trypsin	35 mg.	25 mg.	10 mg.	2 × 10 mg.
Chymotrypsin	35 mg.	25 mg.	10 mg.	2 × 10 mg.
Carboxypeptidase	>25 mg.			
Papain	35 mg.			
Ficin	35 mg			
Human plasmin, Kline fract. A + SK	1000 units	500 units		350 units

acutely tolerated doses, but little or no blood-pressure change occurred when such a dose was given in a four-hour infusion. Pretreatment with diphenhydramine hydrochloride (Benadryl), 5 mg./kg., did not alter the hypotensive effect of fast infusions. No significant ECG changes were observed except for occasional T-wave reversal with crude pancreatic protease. FIGURE 17 shows an example of the effect of trypsin, 10 mg./kg., given twice daily for five days to a rabbit with a radioactive marginal ear vein clot and pulmonary emboli. Injections were given intravenously into the contralateral ear. No decrease in radioactivity of the clot occurred. There was

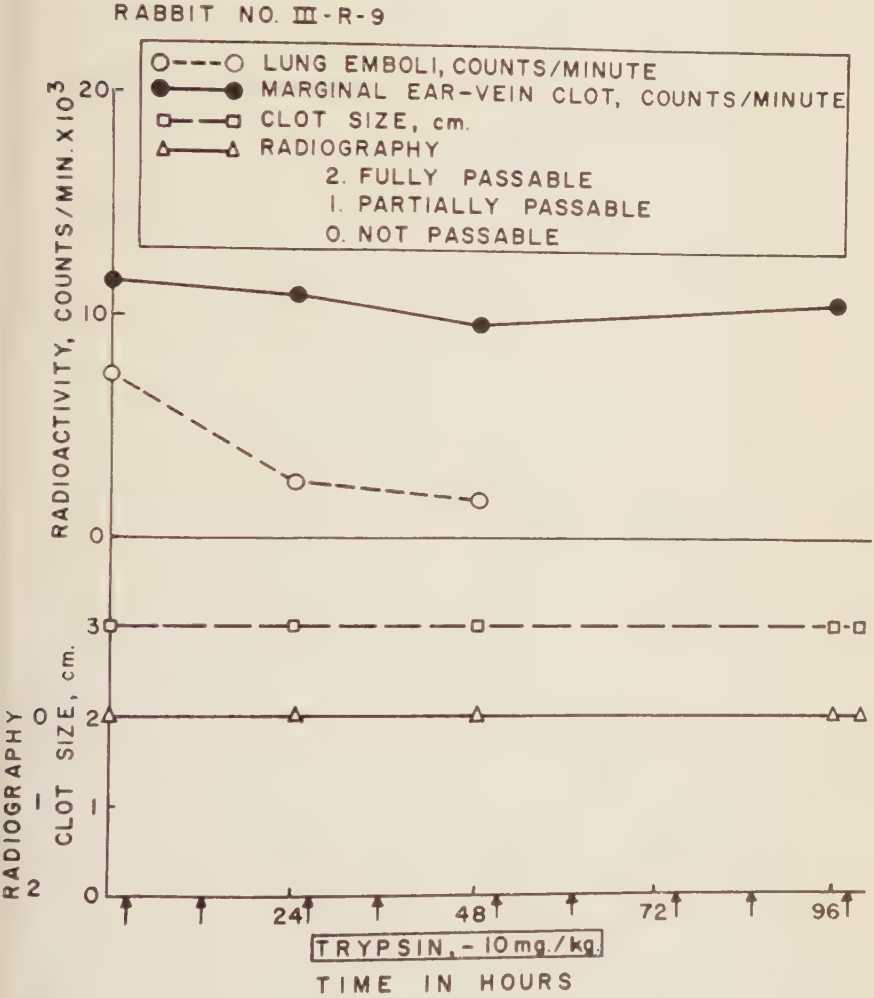

FIGURE 17. Radioactivity of I¹³¹-labeled marginal ear vein clot and pulmonary emboli in a trypsin-treated rabbit. Radiographic and transillumination data.

TABLE 2
FIBRINOLYTIC AND SIDE EFFECTS OF VARIOUS ENZYME PREPARATIONS

Enzyme preparations	Species	No. of admin. × dose range in mg. or units/kg. I.V.*	+ = effective, 0+ = occasionally effective, 0 = no effect, − = decrease, $\underline{0}$ = occasional decrease, H = hemorrhage, OH = occasional hemorrhage						
			Fibrinogen level	Effect on clotting index	Blood pressure infusion rate		Histopath.	*In vivo* anticoagulant†	Lysis of clot‡
					fast	slow			
Crude pancreatic protease	Dog	1 × 25 mg.	−	−	−	0	H	+	0
Trypsin	Dog Rabbit	1–5 × 10–35 mg.	−	−	−	0	H	+	0
Chymotrypsin	Dog Rabbit	1–5 × 10–35 mg.	−	−	−	0	H	+	0
Carboxypeptidase	Dog	1 × 25 mg.	0	0	0	0	0	0	0
Papain	Dog	1 × 35 mg.	−	−	−	0	H	+	0
Ficin	Dog	1 × 35 mg.	−	−	−	0	H	+	0
Varidase (SK-SD)§	Dog	1–5 × 100,000–1,000,000 units	0	0	$\underline{0}$	0	0	0	0

* Plasmin: Loomis units. Streptokinase: Christensen units.
† Prevention or delay of obstruction of canalized clots.
‡ This column includes data from animal experiments only.
§ SK = streptokinase; SD = streptodornase.

TABLE 2 (*Continued*)

Enzyme preparations	Species	No. of admin. × dose range in mg. or units kg. I.V.*	+ = effective, 0+ = occasionally effective, 0 = no effect, – = decrease, $\underline{0}$ = occasional decrease, H = hemorrhage, OH = occasional hemorrhage						
			Fibrinogen level	Effect on clotting index	Blood pressure infusion rate		Histo-path.	*In vivo* anticoagulant†	Lysis of clot‡
					fast	slow			
Bovine plasmin (chloroform-activated)	Dog Monkey Man	1–5 × 15–30 units	$\underline{0}$	$\underline{0}$	$\underline{0}$	0	0	0+	+
Human plasmin preparations Acid extract of Cohn's fract. III + SK	Dog Monkey Man	1–5 × 15–30 units	0	0	0	0	0	+	+
Fletcher's prep. + SK	Dog	1–5 × 250–335 units	–	–	–	0	0	+	+
Kline fract. A + SK	Dog Rabbit Monkey	1–5 × 5 units 1–5 × 15–30 units 1–5 × 60–1000 units	0 0 –	0 0 –	0 $\underline{0}$ –	0 0 0	0 0 OH	0 0+ +	0 + +
Kline fract. A + SK, alcohol-purified	Dog Monkey Man	1–5 × 5–30 units	0	0	0	0	0	0+	+
Kline fract. A + SK, alcohol- and acid-purified	Dog Monkey Man	1–5 × 5–30 units	$\underline{0}$	$\underline{0}$	$\underline{0}$	0	0	0+	+

* Plasmin: Loomis units. Streptokinase: Christensen units.

† Prevention or delay of obstruction of canalized clots.

‡ This column includes data from animal experiments only.

no visible change in the length of the clot on transillumination nor alteration of the passability to X-ray contrast media. A gradual decrease of pulmonary radioactivity was evidenced; this was comparable to that found in control emboli (FIGURE 7).

Varidase (streptokinase-streptodornase) and carboxypeptidase exhibited no fibrinolytic or anticoagulant effect, nor did they alter blood-clotting factors. Slight hypotension was sometimes noted with fast infusions of large doses of Varidase. It should be stated that these two enzymes did not receive a fair trial. Streptokinase is known to be a poor activator of canine plasminogen, although highly effective with human plasminogen. Because of the limited supply and the low toxicity of carboxypeptidase, its maximal tolerated dose could not be established. The dose given was chosen to be in the range of the other enzymes on a mg./kg. basis. All enzymes were tested for fibrinolytic activity *in vitro*, using the procedure devised for plasmin assay. Carboxypeptidase and Varidase were the only ones showing no activity even in the highest doses permitted by their solubility.

All plasmin preparations were able to lyse clots at doses above 5 units/kg. Doses above 30 units/kg. caused hypotension, pronounced fall in fibrinogen level, prolongation of prothrombin time, and occasional incoagulability with hemorrhage.

TABLE 3
ANALYSIS OF VARIANCE OF HUMAN PLASMIN (KLINE A)
ALL DOSE LEVELS VERSUS CONTROL
I^{131}-Labeled Clot Experiments in Dogs. 4 Hours After Treatment

Source of variation	D.F.	S.S.	M.S.	F ratio
Among treatments	9	8.62	0.95	3.2 < **
Within treatments	51	15.24	0.29	
Total	60	23.86		

** $P < 0.01$.

P Scores for Human Plasmin at Individual Dose Levels

Dose units/kg.	No. of clots	*P* score
1000	6	<0.001
750	6	<0.001
500	6	<0.001
250	3	<0.001
125	3	N.S.
60	3	<0.5
30	3	<0.001
15	3	<0.001
5	3	N.S.

Study of the Fibrinolytic and Side Effects of Plasmin; Development of Resistance

TABLE 3 shows the analysis of variance of the level of radioactivity of clots 4 hours after treatment with various doses of Kline's human plasminogen fraction A activated with streptokinase, as compared to controls. TABLE 4 presents similar data for the same clots 24 hours later. Treatment was repeated daily; TABLE 5 analyzes the significance of all data obtained within 96 hours. It appears that significant *in vivo* fibrinolysis occurs with all doses above 5 units/kg. Follow-up of vessels after disappearance of the labeled clots revealed that often re-formation of a fresh, nonradioactive clot followed, sometimes several days after termination of plasmin therapy. This may be due to injury to the vessel wall by the presence of the primary clot and by the daily manipulation necessary to measure radioactivity and other data. Occasionally re-formation was seen after lysis of the primary clot, but during the five-day treatment period. This may probably be explained by development of resistance to plasmin (see below). Heparinization during or after plasmin therapy prevented re-formation of clots. Similar results were obtained with a small series of labeled clots placed into the coronary vessels by cardiac catheterization (Ambrus, Back, Ambrus, and Byron, 1956).

TABLE 4
ANALYSIS OF VARIANCE OF HUMAN PLASMIN (KLINE A)
ALL DOSE LEVELS VERSUS CONTROL
I^{131}-Labeled Clot Experiments in Dogs. 24 Hours After Treatment

Source of variation	D.F.	S.S.	M.S.	F ratio
Among treatments	9	4.69	0.52	2.0*(*)
Within treatments	46	11.88	0.26	
Total	55	16.57		

*(*) $P = 0.05$.

P Scores for Human Plasmin at Individual Dose Levels

Dose units/kg.	No. of clots	*P* score
1000	6	<0.01
750	3	<0.50
500	3	<0.01
250	3	<0.20
125	3	N.S.
60	3	<0.50
30	3	<0.02
15	3	<0.02
5	3	N.S.

TABLE 5
ANALYSIS OF VARIANCE OF HUMAN PLASMIN (KLINE A)
ALL DOSE LEVELS VERSUS CONTROL
I^{131}-Labeled Clot Experiments in Dogs. Up to 96 Hours After Treatment

Source of variation	D.F.	S.S.	M.S.	F ratio
Among treatments	8	1.91	0.239	2.81**
Within treatments	37	3.14	0.085	
Total	45	5.05		

** $P < 0.05$.

P Scores for Human Plasmin at Individual Dose Levels

Dose units kg.	No. of clots	*P* score
1000	3	<0.25
500	3	0.01
250	3	<0.10
125	3	<0.10
60	3	<0.10
30	3	<0.01
15	3	<0.05
5	3	N.S.

In all the experiments discussed so far, treatment was begun half an hour after forming the labeled clots. The question arose whether plasmin treatment would be effective in lysing older, organized clots. In a series of experiments (Back, Ambrus, Byron, and Shulman, 1956; Back, Ambrus, Simpson, and Shulman, 1957) labeled clots were produced in several veins of dogs at various time intervals. Thus, clots of various ages could be treated simultaneously in the same individual. FIGURE 18 charts radioactivity in a dog carrying 3-day-, 2-day-, 1-day-, and half-hour-old clots. FIGURE 19 shows results in a dog with two 5-day-old clots. Clots that had been present for less than 3 days disappeared completely during a 5-day treatment with daily infusions of 30 units kg. of preparation 9 (acid extract of human fraction III plus SK), while 5-day-old or older clots did not change significantly.

Four mechanisms seem to be involved in the resistance of older clots to plasmin therapy:

(1) By the third day, the ends of the clot, the areas of detachment from the vessel wall, and all loosely attached groups of fibrin were covered with endothelial cells. By the end of the tenth day clots were recanalized and were lined with endothelial covering. This endothelialization probably prevented contact between the fibrin fibers and the circulating plasmin.

(2) The clot gradually became a more homogeneous and dense mass, with

increasing numbers of fibroblasts. This structure may be less amenable to fibrinolytic therapy.

(3) By the tenth day collagen fibers appeared in the clot; it is known that such fibers are not attacked by plasmin.

(4) The factors mentioned above do not explain the occasional re-formation observed during the five-day period of treatment. It became evident that most of the lysis occurred within twenty-four hours after the first treatment, while subsequent treatments had much less relative effect on the remaining clot. The question arose whether an increase in antiplasmin level could be involved. Antiplasmin levels are indicated in FIGURES 18 and 19. In these experiments a definite increase in antiplasmin occurred after the first treatment, coinciding with less fall of clotting index and fibrinogen level following the administration of plasmin. While these studies are not as yet completed, on the basis of available data (Back and Ambrus, 1956) it appears

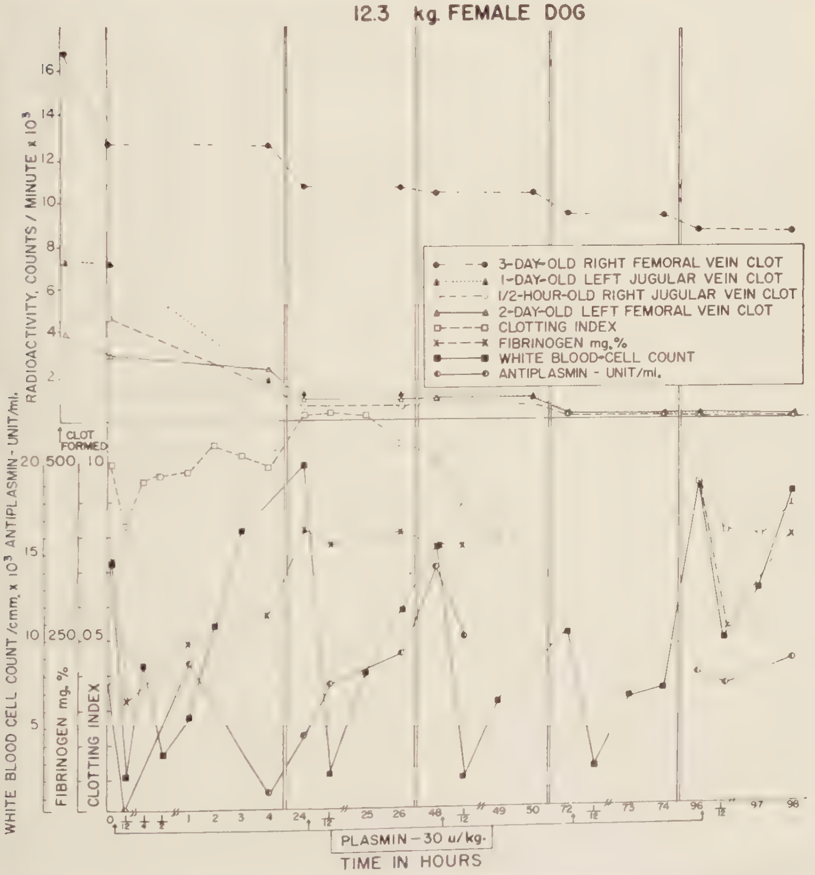

FIGURE 18. Lysis of I¹³¹-labeled clots of various ages in dog treated daily with plasmin, 30 units/kg.

that plasmin evokes fluctuations in the antiplasmin level, but that these ar
not always sufficient to explain resistance to plasmin therapy.

In experimental animals the main side effects of plasmin were hypotensio
and decrease in fibrinogen and prothrombin levels. FIGURE 20 shows tha
a certain dose of streptokinase-streptodornase or of plasminogen (Kline'
fraction A) had little hypotensive effect, but if the former activated the
latter to plasmin, pronounced hypotension occurred. FIGURE 21 show
hypotensive responses to rapid infusions of various doses of similar preparations. No hypotension occurred up to 15 units/kg.; only slight and fleeting hypotension with 30 units/kg.; and definite hypotension, proportiona
to the dose, with higher doses.

A decrease in fibrinogen and prothrombin level occurred with all plasmi
preparations in doses above 30 units/kg.; at the 30 units/kg. dose level the
response was variable; for example, Kline's fraction A plus SK produce
little or no change, while the acid extract of Cohn's fraction III plus SK
FIGURES 18 and 19) usually caused a moderate fall.

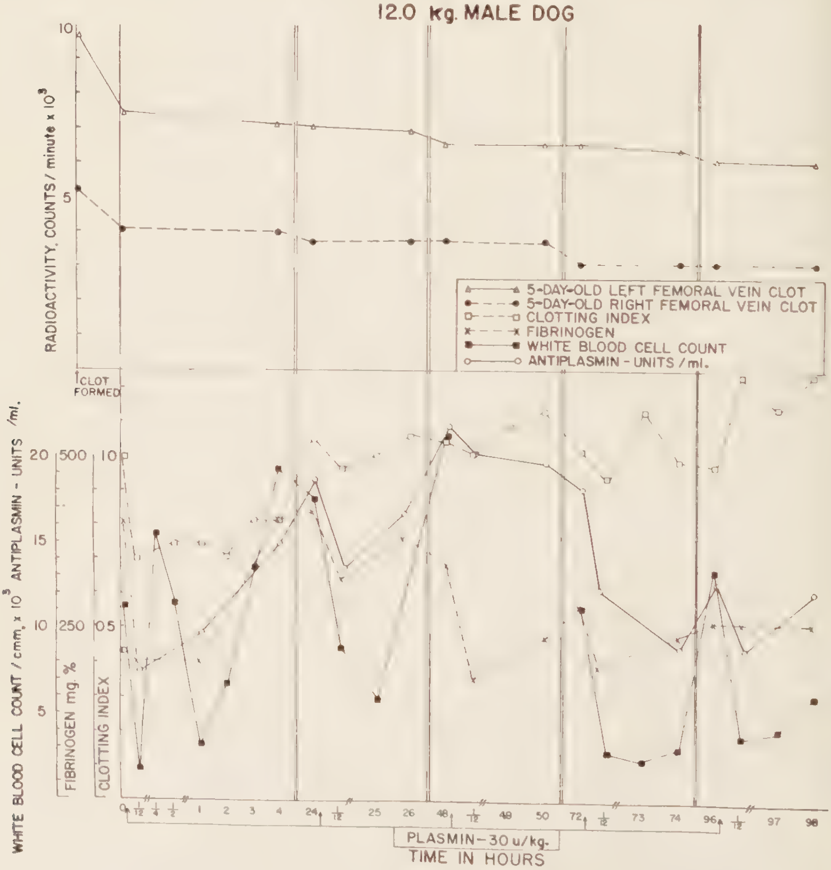

FIGURE 19. Effect of plasmin, 30 units/kg., on 5-day-old I^{131}-labeled clots in dog.

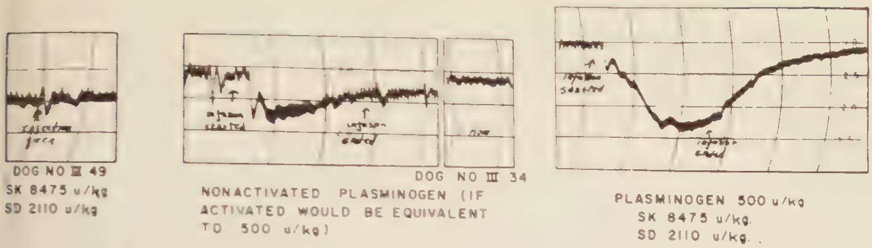

FIGURE 20. Effect of streptokinase-streptodornase, nonactivated and activated plasminogen on blood pressure.

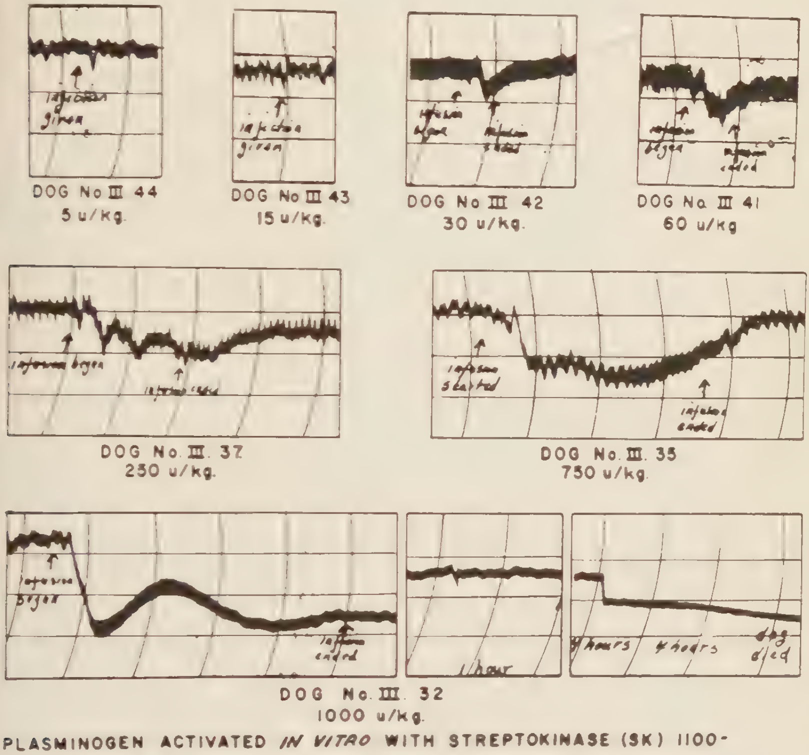

FIGURE 21. Effect of various doses of plasmin on blood pressure of dogs.

All plasmin preparations produced leukopenia, followed by leukocytosis, and thrombocytopenia. These effects did not change appreciably during a five-day treatment period (see FIGURES 18 and 19) in spite of the development of resistance to other effects.

TABLE 6 compares pathological findings in dogs receiving plasmin (30 to 1000 units/kg.) and trypsin (35 mg./kg.) daily for 5 days. Of the 13 trypsin-treated dogs, 11 died; of the 15 plasmin-treated dogs, 4 died. In the latter, plasmin effects appeared not to be the main cause of death. All others

Table 6

Gross and Microscopic Pathological Data of Dogs Receiving Daily Intravenous Infusions of Trypsin and Plasmin for 5 Days

		Trypsin 35 mg./kg.	Plasmin 30 to 1000 units/kg.
Total no. of dogs		13	15
No. died spontaneously		11	4
Lung	Gross	Hemorrhagic patches	Only few hemorrhagic
	Microscopic	Marked congestion, atelectasis alveolar capillaries dilated, edema	Focal hemorrhage associated with atelectasis, edema
Heart	Gross	Normal	Normal
	Microscopic	Focal hemorrhage (one myocardial degeneration)	Mild congestion
Liver	Gross	Some hard, enlarged, dark	Normal
	Microscopic	Several sinusoidal congestion, mild focal hemorrhage	Marked sinusoidal congestion with mild focal hemorrhage
Spleen	Gross	Few with hemorrhagic nodules	Enlarged
	Microscopic	Sinusoidal congestion with focal hemorrhage, phagocytes with pigment	Marked congestion of red pulp
Stomach	Gross	1 with multiple ulcers, 1 with large single ulcer	Normal
	Microscopic	1 with hemorrhage beneath muscularis mucosa	—
Intestine	Gross	Hemorrhagic sacs on external surface	Normal
	Microscopic	Hemorrhage in muscularis extending out to serosa	Mucosal congestion
Kidney	Gross	Hyperemic	Normal
	Microscopic	Mild degree of glomerular congestion	Medullary congestion with extravasation of blood
Adrenal	Gross	—	—
	Microscopic	—	Marked medullary congestion
Brain	Gross	Normal	Normal
	Microscopic	Focal hemorrhages in one	Normal
Pituitary	Gross	—	Slightly hemorrhagic
	Microscopic	—	Congestion of sinusoids (mild)
Blood at time of death		Incoagulable	Coagulable

were sacrificed on the fifth day. Pathological findings in dogs that died during treatment with other proteolytic enzymes were similar to these found with trypsin.

Clinical Studies

To date 19 patients have been treated with 1 to 5 doses of one or more preparations of plasmin. Table 7 summarizes side effects of the most frequently employed preparations. Tables 8 and 9 show examples of clinical and hematologic changes in two patients. The most disturbing side effects were chills: severe chills occurred in all patients treated with preparation 9 (acid extract of Cohn's fraction III plus SK); definite to mild chills were observed with about half of the treatments employing preparation 12 (Kline's human plasminogen fraction A plus SK, alcohol-purified); and slight chills were noticed with about half of the treatments with preparation 13 (Kline's human plasminogen fraction A plus SK, alcohol-purified and acid-treated).

Table 7

Clinical Side Effects of Various Plasmin Preparations

Source of plasminogen and method of preparation	Activator	Purification	Chills, hyperthermia, increase BMR	Hypotension	Leukopenia thrombocytopenia, fibrinogenopenia, prothrombinopenia
Bovine (Loomis)	Chloroform	–	–	–	+
Human, acid extract of Cohn's fraction III	SK	–	++	±	+
Human, fraction A of Cohn's fraction III (Kline)	SK	Alcohol (Kline)	50% +	±	+
		Alcohol and acid (Kline)	50% ±	±	+

Table 8

Patient W. G. II.5

	Time	Thrombocytes	WBC	RBC	Hematocrit	Blood pressure	Temperature	BMR	Pulse min.
	0	280,000	6,300	3,340,000	30 %	105/55	100° F.	+10	100
Infusion of 10 units/kg. acid extract of Cohn's fraction III	40 min.	48,000	4,350	2,580,000	24 %	75/50	99° F.		96
	1 hr.	40,000	800	2,730,000	28	110/70	100.6° F.		92
	2 hr.	30,000	700	2,720,000	27 %	74/50	103.2° F.	+52	132

Table 9

Patient T. B. I.2

Time		Thrombocytes	WBC	RBC	Hematocrit	Hemoglobin	Blood pressure	Temperature	Pulse/min.
	0	130,000	8,500	4,000,000	39 %	13.2 gm.	210/100	97° F.	60
Infusion of 30 units/kg. acid extract of Cohn's fraction III	1 hr.	100,000	3,150	3,500,000	38 %	12.8 gm.	210 100	97° F.	64
	2 hr.	100,000	3,250	3,550,000	36 %	12.8 gm.	210 85	98.2° F.	80
	3 hr.	60,000	3,100	3,850,000	39 %	12.7 gm.	215 80	98.5° F.	80
	5 hr.	50,000	6,550	3,780,000	36 %	12.8 gm.	215 80	101.2° F.	80
	24 hr.	90,000	11,400	3,840,000	39 %	12.8 gm.	180 74	97° F.	72

No chills occurred with chloroform-activated bovine plasminogen and non-activated human plasminogen (Kline's fraction A). Hyperthermia was usually proportional to the degree of chills. Temperature increases usually ranged from 2° to 5° F., but with preparation 9 occasional increases as great as 8° F. were observed. Hyperthermia usually lasted 2 to 6 hours. Increase in pulse rate was proportional to increase in body temperature. Chills and hyperthermia were not prevented or suppressed by the intramuscular administration of 25 to 50 mg. chlorpromazine or of 25 to 50 mg. Benadryl, by intravenous injections of 10 mg. morphine or of 0.5 gm. calcium gluconate, or by combinations of these agents. Pretreatment with aminopyrene appeared to be of some value. Most often 0.3 gm. was given orally 2 hours before treatment and an additional 0.3 gm. when about half of the plasmin dose was infused. In one case, 0.3 gm. aminopyrene given every 4 hours for 24 hours before treatment failed to prevent chills and hyperthermia produced by preparation 9. Intravenous Amytal Sodium or Pentothal Sodium in doses sufficient to produce light anesthesia promptly arrested even severe chills, but did not prevent the eventual development of hyperthermia.

The BMR was measured before treatment after an intravenous infusion of saline had been started, and at various time intervals during and after plasmin therapy. A gradual increase in BMR was noticed during plasmin therapy before the patient complained of discomfort or cold and long before chills and hyperthermia occurred. Amytal or Pentothal anesthesia did not prevent or alter the increase in BMR. In general, the degree of increase in the BMR was proportional to the severity of chills and to the hyperthermia.

Hypotension occurred occasionally with all the human plasmin preparations. It was usually not severe, and only in few cases treated with preparation 9 was it necessary to administer Levophed.

Leukopenia, followed by leukocytosis, and thrombocytopenia occurre with all preparations. No significant changes were found in red-blood-cel count in the hematocrit or in hemoglobin values.

Tables 10 and 11 show changes in blood clotting and in fibrinolytic factors Decrease of the prothrombin level was frequently seen. Decrease of th plasminogen level was interpreted as being indicative of the activation o the patient's own fibrinolysin system. Antiplasmin levels decreased durin

TABLE 10

Patient W. G. II.5

Time		Plasmin assay Lysis time at 28° C.	Anti-plasmin units/ml.	Plasminogen units/ml.	Fibrinogen mg. %	Prothrombin time in seconds	Lee-White clotting time
Infusion of 10 units/kg. acid extract of Cohn's fraction III	0	neg. in 72 hr.	22	22	425	16	7 min. 30 sec.
	40 min.	24 hr.	18	20	319	23	9 min. 33 sec.
	1 hr.	24 hr.	7.2	13.3	298	17	9 min. 30 sec.
	2 hr.	48 hr. partial	18	8	383	17	8 min. 30 sec.

TABLE 11

Patient T. B. I.2

Time		Plasmin assay Lysis time at 28° C.	Anti-plasmin units/ml.	Plasminogen units/ml.	Fibrinogen mg. %	Prothrombin time in seconds
	0	neg. in 72 hr.	24	30	322	17.6
Infusion of 30 units/kg. acid extract of Cohn's fraction III	1 hr.	24 hr.	20	25	262	15.0
	2 hr.	24 hr.	18	17	222	16.5
	3 hr.	24 hr.	19	3.2	180	17.5
	5 hr.	48 hr. partial	32	2.6	284	17.5
	24 hr.	48 hr. partial	30	32	304	18.5

plasmin infusion and, in certain cases, showed a rebound increase following completion of treatment. Increase of the level of free plasmin could be demonstrated, but in most instances it was too small to be quantitated. Instead of plasmin units per ml., TABLES 10 and 11 indicate lysis time at 28° C. The test was performed similarly to the plasmin assay at 45° C., except for the lower temperature used to prevent inactivation of plasmin during the longer test period. Tubes were observed for 72 hours; normal plasmas did not produce lysis within this period of time.

Because of the small number of patients and the widely varied nature of the conditions treated, no general conclusions can be drawn as to the therapeutic value of the various plasmin preparations. It appears, however, that thromboses of long standing are unresponsive to plasmin therapy. In animal experiments, clots were effectively lysed by doses of plasmin greater than 5 units/kg. In certain clinical cases of acute thrombosis, therapeutic results were obtained with doses as low as 5 units/kg. An illustrative case history follows. A detailed report of clinical studies will be published elsewhere.

Case report. A 34-year-old white housewife with far-advanced carcinoma of the stomach and intraperitoneal dissemination developed acute thrombophlebitis of the left leg. The extremity was obviously swollen and mildly cyanotic. Homan's sign was positive, and the superficial veins were distended. Arterial pulses were normal. The circumference of the left calf was 12 cm. greater than that of the right, of the left lower thigh 4 cm. greater than that of the right. The cuff compression test of Lowenberg (1954) was strongly positive, both at the calf and the thigh.

Plasmin therapy was started about 24 hours after the onset of thrombophlebitis. Five units/kg. of preparation 9 were infused over a 2-hour period, followed by 5 units/kg. of preparation 13 (activated with preparation 12 instead of SK). In spite of premedication with aminopyrene, 0.6 gm., and chlorpromazine, 50 mg., the patient developed discomfort, chills, and fever during the infusion. Chills were arrested by intravenous administration of Pentothal Sodium, but the temperature continued to rise, reaching 103.2° F. The BMR reached +117. The reaction was followed by profuse diaphoresis, and the temperature returned to normal. The pulse rate reached 160, but the blood pressure remained normal.

By the following morning, the color of the left leg was almost normal. The circumference of the left calf was 6.5 cm. greater than that of the right; of the left thigh, 3 cm. greater than that of the right. The cuff test was still positive.

A second infusion, containing 5 units/kg. of preparation 12 and 10 units/kg. of preparation 13, was administered over a $1\frac{1}{2}$-hour period that afternoon. There was a mild febrile reaction to this preparation, with slight chills, moderate discomfort, and maximum temperature of 101° F.

By the next morning, the color of the left leg was normal. The circumference of the left calf was 5 cm. greater than that of the right; the circumference of the left thigh was 3 cm. greater than that of the right. Homan's sign was negative. The cuff test was weakly positive. Two days later, with no further therapy, the left leg appeared entirely normal. Homan's sign was negative. The cuff test was negative. The circumference of the left calf was 3 cm. greater than that of the right; of the left thigh, 1 cm. greater.

The patient's general condition deteriorated rapidly and she died a few days later. At autopsy, a relatively fresh thrombus was found in the left iliac vein.

Comment. The course of this patient suggests that an acute venous thrombosis was successfully treated with two plasmin infusions, 24 and 56 hours after onset. Thrombosis recurred several days later. Under ordinary circumstances, anticoagulants would have been administered immediately after completion of plasmin therapy. In this terminal patient, who was bleeding actively from a gastric carcinoma, this was not practicable. Recurrence of thrombosis several days after completion of treatment is an additional indication that clot lysis during therapy was not fortuitous.

In Vitro *Experiments*

On the basis of clinical studies it appeared that preparations with less SK activity are less toxic. Experiments were undertaken to compare the biochemical and immunological characteristics of these various preparations.

Rabbits were immunized with subcutaneous doses (500 units/kg.) of the various plasmin preparations, given three times every other day. Two weeks later the serum was tested for antistreptokinase activity. TABLE 12 summarizes these results and compares the capacity to produce antistreptokinase and the activator activity of various plasmin preparations. In general, the better antistreptokinase producers were the more potent activators. Only a preparation activated with human lung mitochondria exhibited some acti-

TABLE 12

ABILITY OF VARIOUS PLASMIN PREPARATIONS TO PRODUCE ANTISTREPTOKINASE ANTIBODIES IN RABBITS AND TO ACTIVATE BOVINE PLASMINOGEN *IN VITRO*

Plasminogen and method of preparation	Activator	Purification	Antistreptokinase production	Bovine plasminogen activation
Bovine (Loomis)	Chloroform	—	—	—
Human, acid extract of Cohn's fraction III	SK	—	++	++
Human, fraction A of Cohn's fraction III (Kline)	SK	—	++	++
	SK	alcohol (Kline)	+	++
	SK	alcohol and acid (Kline)	±	−
	SK	alcohol and acid (Kline); SK added after activation	++	+
	Spontaneous	—	—	—
	Spontaneous, SK added after activation	—	++	++
	Human lung mitochondria	—	—	+
	Human lung mitochondria; SK added after activation	—	++	++
—	SK	—	++	—

SK = streptokinase.
++ = strong effect.
+ = weak effect.
± = trace or variable effect.
− = negative.

TABLE 13

SPECIFICITY FOR FIBRIN VERSUS FIBRINOGEN OF VARIOUS PLASMIN PREPARATIONS

Decrease in fibrinogen level of a 0.3% bovine fibrinogen solution after incubation at 45° C. for the length of time in which the same quantity of the same preparation would completely lyse a 0.3% fibrin clot. Values indicate mean of three experiments. SK = streptokinase.

Plasminogen and method of preparation	Activation	Purification	Percentage decrease in fibrinogen level as compared to control Loomis units of plasmin	
			0.5	1
Bovine (Loomis)	Chloroform	—	67	79
Human, acid extract of Cohn's fraction III	SK	—	5	10
Human, fraction A of Cohn's fraction III (Kline)	SK	Alcohol (Kline)	9	13
Human, fraction A of Cohn's fraction III (Kline)	SK	Alcohol and acid (Kline)	64	80

vator activity without antistreptokinase-producing potency. Preparations with less clinical side effects were weaker activators and less potent antistreptokinase producers. Spontaneously activated plasmin had no activator activity. Upon addition of SK, high activator activity and anti-SK producing ability appeared. Preparation 13, in which the SK used for activating the plasminogen was inactivated by acidification, had no activator activity and only trace anti-SK producing potency in rabbits. When SK was added to this preparation, anti-SK producing ability and activator activity appeared. Increase in activator activity, however, was weaker than with spontaneous plasmin plus SK, or human lung-mitochondria activated plasmin plus SK.

On the basis of the above experiments the following hypothesis may be proposed. SK activates plasminogen and forms a complex with plasmin. This complex possesses activator activity. Alcohol purification removes free SK from the preparation, thus reducing somewhat its anti-SK forming ability, but without affecting the SK plasmin complex or the activator activity. Acid treatment inactivates the SK in the complex, activator activity disappears, and only trace anti SK forming activity remains. When SK is added to spontaneously activated plasmin, activator and anti-SK producing activity appear. When SK is added to preparation 13 (SK-activated and acid-treated plasmin), anti-SK producing ability reappears but, since some inactivated SK occupies positions on the plasmin molecule, less active SK can enter into the complex with plasmin and the resultant activator activity is weaker.

TABLE 13 shows an experiment aimed at determining the specificity of various plasmin preparations for fibrin. Plasmin was assayed as in the previous studies, except that the end point was complete lysis of the clot. In the experiments shown, 0.5 and 1 unit of plasmin, respectively, were used. The minimal amount of lima-bean trypsin inhibitor that could completely inhibit one Loomis unit of the various preparations of plasmin was determined. A series of tubes was set up as in the plasmin assays, except that no thrombin was added. At the time when the parallel plasmin assay tubes showed complete lysis, lima-bean inhibitor was added to the unclotted tubes, and they were placed in an ice bath. The fibrinogen level was determined before and immediately after arresting the reaction. Preparations more specific for fibrin than fibrinogen are expected to digest less fibrinogen in the time necessary to lyse a clot produced with the same amount of fibrinogen, than preparations that are less specific for fibrin. It appears from TABLE 13 that chloroform-activated bovine plasmin and preparation 13 (alcohol- and acid-purified human plasmin) destroyed 64 to 80 per cent of a 0.3 per cent fibrinogen solution in the time needed to lyse a 0.3 per cent fibrin clot. On the other hand, preparations 9 and 12, both containing SK, digested only 5 to 13 per cent of fibrinogen under the same conditions. It thus appears that SK-containing preparations are more specific for fibrin than preparations of equal fibrinolytic activity containing no SK.

DISCUSSION

On the basis of animal experiments with I^{131}-labeled clots, plasmin preparations appeared to be the most promising of all the proteolytic enzymes tested as *in vivo* fibrinolytic agents. Doses that effectively lysed clots without producing significant changes in coagulation factors or toxic side effects were found. Most of these preparations induced chills, hyperthermia, increase in BMR, and occasional hypotension in patients. In animal experiments an inverse relationship between the age of the clot and the success of plasmin therapy was found. This was confirmed by a limited clinical trial. It thus appears that an important field of application of intravenous plasmin therapy would be acute venous and arterial thrombosis of less than seventy-two hours' duration. The most important clinical disorder in which plasmin might play a therapeutic role is coronary thrombosis. In this condition the side effects mentioned above represent serious contraindications. In an attempt to produce preparations with fewer side effects a number of different types of plasmin preparations were examined. Toxicity appeared to be associated with streptokinase content. Human plasminogen or chloroform-activated bovine plasmin showed no significant side effects. Preparations containing less streptokinase were less toxic. On the other hand, elimination of streptokinase decreased the specificity of these preparations for fibrin. It is felt that preparations such as Kline's human plasminogen fraction A plus SK, alcohol-purified, are suitable for the treatment of certain cases of acute peripheral thrombosis. Improved preparations are needed, however, before clinical trials in coronary thrombosis can be attempted.

Evidence was presented that streptokinase may form a complex with plasmin and that this complex may be responsible both for activator activity and specificity for fibrin. Some of the experiments of Sherry and Alkjaersig (1956) seem to point in the same direction.

SUMMARY

(1) Methods for the objective *in vivo* evaluation of fibrinolytic agents have been described. These methods were based on the disappearance of clots produced with I^{131}-labeled fibrinogen.

(2) The following proteolytic enzymes were investigated for fibrinolytic and pharmacological properties: crude pancreatic protease, trypsin, chymotrypsin, carboxypeptidase, papain, ficin, Varidase, chloroform-activated bovine plasmin, and various preparations of human plasmin. Only the plasmin preparations showed *in vivo* fibrinolytic activity at nontoxic dose levels.

(3) Plasmin (15 to 30 units/kg.) effectively dissolved experimental *in vivo* clots less than 3 days old, but was ineffective against clots older than 3 days.

(4) In clinical studies, therapeutic results were obtained with plasmin (5 to 30 units/kg.) in acute peripheral thrombosis, but no effect was seen in arterial or venous thrombosis of more than five days' duration. The clinical material at present is too small to draw general conclusions as to the therapeutic value of plasmin.

(5) The most important side effects seen in patients were chills, hyperthermia, increase in BMR, and hypotension. Chills could be arrested by intravenous Amytal Sodium or Pentothal Sodium. Pretreatment with aminopyrene, chlorpromazine, and other agents had only a limited value in preventing these reactions. Hematological changes included leukopenia followed by leukocytosis, thrombocytopenia, fibrinogenopenia, and prothrombinopenia. These were usually minor in degree, and rapid return to normal occurred.

(6) A number of plasmin preparations were studied. Those with little or no streptokinase content produced fewer side effects in patients.

(7) Immunological and *in vitro* studies indicated that plasminogen activator activity is associated with the streptokinase content of the preparations. Preparations containing streptokinase showed more specificity for fibrin, as opposed to fibrinogen, than did preparations not containing streptokinase. Evidence is presented in favor of the concept that human plasmin may form a complex with streptokinase and that this complex may be responsible for activator activity.

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Discussion of the Paper

QUESTION: I should like to ask the authors if they would be kind enough to enlarge slightly on the comment that antiplasmin was not responsible for the failure to react in the second or third day. The reason I ask this is that, if one were trying merely to describe the behavior of the inhibitor, one would find exactly what you have found by stimulating an animal. At zero hours the inhibitor falls and in twenty-four hours there are very high levels. This has been interpreted by some as being a defense mechanism. Indeed, is this not a defense mechanism that makes your medication ineffective, and would that not explain the results?

J. L. AMBRUS (*Roswell Park Memorial Institute, Buffalo, N. Y.*): N. Back in our laboratory has just finished a series of investigations on these points. If he takes dogs either with or without clots and treats them daily with plasmin, he finds very pronounced fluctuations. Some dogs will behave very nicely. They will show some decrease in antiplasmin level during the first twenty-four hours after infusion. Later the level will be higher than the base line, and it may come down and may fluctuate during each daily treatment. On the other hand, in this series there are also dogs that show this same fluctuation, but in which the antiplasmin level is actually below that which we find in normal dogs or below the initial level of the same dog. Yet in these dogs we have the same development of tolerance as in the dogs whose antiplasmin is usually higher than their base line. In other words, development of tolerance does not correlate with increase in antiplasmin levels. There may be some technical difficulty that we may be able to correct eventually, but at present we can conclude only that, while antiplasmin changes occur, they are not the main factors explaining resistance in all dogs.

M. SIEGEL (*Arthur P. Noyes Institute for Neuropsychiatric Research, New*

Hampshire State Hospital, Concord, N. H.): I should like once more to point out the contamination of bovine thrombin with plasminogen and the great danger of comparing fibrinolytic and fibrinogenolytic titer, since in fibrinolytic determinations you are adding a bovine thrombin preparation that is richer in bovine plasminogen than the commercial preparation of bovine plasminogen. I wonder if this contaminant might not have something to do with time susceptibility of your clots in the dog. You are starting out with a clot very rich in bovine plasminogen. With time this plasminogen may be washed out so that eventually your clot is exposed only to the circulating plasmin, not to any local high concentration of bovine plasmin.

J. L. Ambrus: We are aware of the danger of using unpurified thrombin. We plan to prepare some purified thrombin and repeat some of these experiments. On the other hand, I should like to mention again that there are many papers that maintain that thrombin itself is a potent fibrinolytic agent. This is a very real source of error that must be considered, particularly in the specificity test *in vitro*. In the *in vivo* experiments about half of the clots consisted of clotted whole blood of the host. This of course contains large quantities of plasminogen. The experiments were designed in this way to reproduce the situation in human pathology as closely as possible. It is questionable whether any additional plasminogen contamination of the quantities of thrombin used added significantly to the plasminogen present in the clot.

Part II. Clinical Studies

PANCREATIC DORNASE AEROSOLS IN BRONCHOPULMONARY DISEASE

By Maurice S. Segal and Merrill Goldstein

Department of Medicine, Tufts University School of Medicine and the Lung Station (Tufts), and Department of Inhalation Therapy, Boston City Hospital, both in Boston, Mass.

An increasing number of enzymes of various origins are being used for enzymatic debridement of fibrinopurulent and mucous secretions in the tracheobronchial tree in pulmonary and other suppurative diseases.[12, 13] The harmful potentialities of these secretions in obstructing airways and acting as a culture medium for infectious microorganisms are evident. The rapid and safe evacuation of these secretions in the simplest possible manner is a problem of great importance.

Evaluation of Effects from the Use of Therapeutic Enzyme Aerosols

Evaluation of the efficacy of this form of therapy is difficult. For the most part, the study of these agents is based upon clinical impressions of the changes in the daily amount and character of the sputum. Certain minimal changes in the viscosity of a patient's sputum will undoubtedly go undetected in a daily examination of a twenty-four-hour sputum collection. Determination of the effectiveness of these agents depends to a large extent upon the patient's subjective response, that is, the ease of raising sputum, the degree of wheezing and dyspnea, and the severity and frequency of cough. The use of the patient's subjective impressions is often a poor and unreliable method of gaining adequate appraisal of medications.

Pancreatic Dornase

Pancreatic desoxyribonuclease (pancreatic dornase*) is prepared from beef pancreas. Desoxyribonucleoprotein is an important constituent of purulent exudate, and it determines the character, viscosity, and amount of sediment of such exudates.[1] Desoxyribonuclease, whether of streptococcal origin (streptodornase) or of pancreatic origin (pancreatic dornase) is a pus-liquefying enzyme and is capable of degrading desoxyribonucleoprotein.[2, 3] The action of pancreatic dornase is primarily one of depolymerization, and little degradatien of desoxyribonculeoprotein occurs.[4] The absence of appreciable amounts of noxious degradation products, which might be absorbed systemically, permits its relatively safe use.

H. G. Reiser and his co-workers[5] have studied the effect of adding pancreatic dornase or trypsin *in vitro* to mixed substrates of pus of nontuberculous origin and pus from mixed tuberculous empyema. The effect of the enzymes was evaluated on the basis of the degree and rapidity of changes in viscosity.

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* Kindly supplied by Merck Sharp & Dohme Research Laboratories, Rahway, N. J.

In nontuberculous pus, pancreatic dornase was more effective than trypsin in producing lysis, causing it within the first minute of contact. Trypsin, which is a potent fibrinolysin, proved more effective than pancreatic dornase in lowering the viscosity of pus from mixed tuberculous empyema, which is rich in fibrinogen and fibrin.

Pancreatic dornase exhibits some fibrinolytic activity, probably due to contamination with small amounts of trypsin and chymotrypsin.[6] These last-mentioned enzymes have, in addition, mycolytic activity, which probably accounts for the effectiveness of pancreatic dornase on mucous and mucopurulent material. Since many enzymes, including streptokinase and streptodornase, are known to be mechanically labile, this aspect of the drug under trial was tested by Reiser.[7] Employing a Vaponefrin glass nebulizer and an oxygen flow rate of 7 liters/min., he vaporized 5.0 cc. of pancreatic dornase solution into an inverted beaker. The droplets that condensed on the walls of the equipment were collected. Pooled pus samples were mixed with this "nebulized" pancreatic dornase and with "non-nebulized" pancreatic dornase and the viscosity was then measured after 1-, 5-, and 15-min. intervals, and after 24 hours. The results indicated that after aerosolization there was no significant change in the lytic activity toward pus of this agent.

From the above pharmacological data, it appears that pancreatic dornase has a specific effect against desoxyribonucleic acid and to a lesser degree against fibrin and mucus. These data suggested that this enzyme might be of value in the treatment of processes in which mucopurulent and viscous secretions are present.

Current Studies

Pancreatic dornase aerosols were employed and evaluated in the management of 100 patients with various pulmonary conditions: bronchiectasis, pulmonary abscess, pulmonary atelectasis, unresolved pneumonia, chronic bronchitis, chronic bronchial asthma, and emphysema. As noted in our preliminary studies,[8] in addition to the chronic disease, most of the patients had one or more complicating processes such as fibrosis, bronchitis, and cardiac failure.

We employed dosages of 50,000 to 100,000 units per treatment, and administered 1 to 4 treatments daily for 1 to 7 days. Clinical improvement, and decrease in cough and in volume of sputum were used as a therapeutic guide for dosage and continuation of therapy. The dornase was generally mixed with 1 or 2 cc. of saline shortly before its administration. When bronchoconstriction was evident, 0.5 to 1 cc. of a potent bronchodilator aerosol (Vaponefrin) was added to the mixture. Pyribenzamine (20 mg.) was added occasionally to alleviate pharyngeal irritation or to prevent potential allergic reactions. Thorough rinsing of the mouth and oropharynx with saline or water after each treatment was encouraged.

Approximately one half of the patients in our study received the aerosols of pancreatic dornase simultaneously with intermittent positive-pressure breathing (IPPB/I) using the Bennett valve,[8, 9] while the other half received the enzyme with the Vaponefrin nebulizer-constant oxygen flow technique or with the Vaponefrin nebulizer-Eliot air pump face mask unit.[15] It appeared to us that better results were obtained with the combined aerosol and intermittent positive-pressure breathing techniques. The latter technique provides more complete ventilation of the lungs and, hence, more complete distribution of the enzyme to poorly aerated segments.[9, 10] The asthmatic patients especially seemed to tolerate the 15- to 20-min. aerosol treatment better when it was given with the Bennett valve apparatus. A number of patients were given pancreatic dornase aerosols just prior to exsufflation with the Eliot Vent-El-Aire unit, which employs alternate positive-negative pressure therapy.[11]

Results

In most patients, beneficial effects were noted within one half to one hour after treatment. The cough became more effective, and increasing amounts of sputum were expectorated in the following 3 to 6 hours. Twenty-four-hour sputum volumes increased to two or three times the control volumes. At first the sputum was thick and gelatinous. Gradually it changed in character and became thin and milky in appearance. It soon lost its yellow and greenish color; a marked decrease in pus cells and cellular elements was subsequently noted microscopically.

Some patients noted subjective relief after the first treatment, and marked clinical improvement was generally noted after one or two days of treatment; in cases with fever the temperature usually dropped to normal; cough was more productive than it had been previously, and secretions were expectorated with greater ease. The patients slept and felt better, and appetite and sense of taste improved, probably due to the decrease in cough and in the amount of sputum swallowed. In patients with chronic bronchial asthma and emphysema in status asthmaticus, the sputum volumes were generally small prior to therapy, and there was a persistent wheeze and a hacking nonproductive cough. Rapid improvement was generally noted after one or two treatments, probably due to the production of a more efficient cough mechanism and more copious sputum. In cases in which a superimposed infection or pneumonitis was present, repeated treatment with pancreatic dornase appeared to speed recovery.

The best results were obtained in patients who had large amounts of tenacious mucopurulent secretions in the tracheobronchial tree and in whom evacuation of these secretions was impeded. Included in this category were the patients with bronchiectasis, infected atelectasis, abscess, and unresolved pneumonia.

Less striking results were obtained in the patients who had chronic asthma or emphysema as an underlying condition, with a superimposed infection putting them in status asthmaticus. The variable amount of obstructing secretions in these patients might account for the variability in results. Those in whom this factor was great would be expected to improve following the liquefaction of their sputum, while those in whom this factor was small would not.

It is much more difficult to evaluate a therapeutic adjuvant that is used only in conjunction with many other forms of treatment than it is to assess the benefit of a therapeutic agent that can be used alone. In our studies pancreatic dornase was not used as the only treatment in any case, and it cannot be credited with a single "cure." The objective evidence of the effect of administration of pancreatic dornase lay in the decrease in viscosity and the increase in volume of the patient's sputum following its use. It was our impression that the patients receiving it improved more rapidly and uniformly than they would have done otherwise.

Side Reactions

We have not observed any significant side effects in our studies. Several patients noted pharyngeal irritation and some "stiffness of tongue" after four or five days of therapy. Oral hygiene after each treatment was stressed. The longest single course of therapy was seven days.

Using pancreatic dornase we did not observe any of the side effects previously reported with Tryptar aerosol, such as hoarseness, eyelid or nasal irritation, or blood-streaked sputum. Nevertheless, because of the protein nature of the former, allergic reactions may possibly occur in allergic or previously sensitized subjects. Concomitant antihistaminic therapy administered orally or in the aerosol "cocktail" mixture may be employed in these subjects.

Frequent cytological studies (Papanicolaou's stains) on the sputa of many of our patients gave no evidence of metaplasia. These studies were made following the report of Farber that cellular metaplasia had been observed in the sputum of patients treated with Tryptar aerosol.[14] The observation of metaplastic cellular changes in laboratory animals following the use of various enzyme aerosols does not necessarily imply potential production of neoplasia in humans. Cellular metaplasia has been noted by many observers in the sputum and in biopsy specimens of patients with chronic bronchitis and various pulmonary disorders in the natural course of the disease.

Summary

(1) Pancreatic dornase appears to be a safe and most helpful adjuvant in the therapy of bronchopulmonary diseases in which tenacious mucopurulent secretions constitute a major factor in the pathological process, and in which evacuation of the secretions is of utmost importance.*

(2) Aerosols of pancreatic dornase were administered in doses of 50,000 to 100,000 units per treatment, usually dissolved in saline, 1 to 4 treatments daily for courses of 1 to 7 days.

(3) Bronchodilator agents were added to the "cocktail" mixture for the relief of bronchospasm and antihistaminolytic agents for the relief or prevention of topical allergic reactions.

(4) The aerosols were administered directly by means of one of the follow

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* Since the preparation of this paper, the following article has appeared, confirming our original observations with pancreatic dornase and the studies reported here. "Pancreatic Dornase Aerosol in Pulmonary, Endotracheal and Endobronchial Disease," by Eugene E. Cliffton, in *Diseases of the Chest,* vol. 30, p. 373, 1956.

ing techniques: (a) the Vaponefrin nebulizer-constant oxygen flow technique: (b) the Vaponefrin nebulizer with Eliot air pump-oropharyngeal face mask unit; (c) simultaneously with intermittent positive-pressure breathing (IPPB/I), using the Bennett or a similar unit; and (d) preceding exsufflation therapy (alternating P-N) with the Vent-El-Aire unit.

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Discussion of the Paper

Question: May I ask what happens if you continue treatment longer than seven days?

M. Segal: We find that the results are usually striking with shorter treatment, and we are not certain about sensitization. From our past experience with proteolytic agents we have felt that if sensitization occurs, it appears after a seven- or eight-day period of repeated use. We do not have any evidence that one actually gets evulsion of mucosa—that is, destruction—if one continues for a longer period of time. Our definite feeling is that, if you do not achieve results in seven days, you should discontinue therapy.

J. M. Miller (*Veterans Administration Hospital, Fort Howard, Md.*): I should like to mention our experience with the more acute cases of pneumonia that have been treated with enzyme therapy. Our patients have been treated first by putting a catheter in the trachea and then injecting the enzymes. We later nebulized the solutions. In our sick patients we have done a tracheostomy, then put the solutions into the tracheobronchial tree. I would stress the value of a three-way treatment of individuals who are severely ill with pneumonia, who are badly anoxic, and in serious difficulty: first, tracheostomy; second, vigorous mechanical suction; and, third, the use of the enzymes, combined with good suction. It would seem that we have in our possession an ideal combination for treating pneumonia. We also have another weapon: the intramuscular injection of one or another of these enzymes. Most of our work has been done with streptokinase, first in impure and later in the purified form. This gives us a method of attacking the pneumonia, not only from the route of the tracheobronchial tree, but also systemically. I am convinced that we can attain better results by placing antibiotics where we want them by the use of a systemic route of SK administration.

W. S. Tillett (*New York University College of Medicine, New York, N. Y.*): We have been interested in the preliminaries of the aerosol use of streptodornase and purified pancreatic dornase, and have found that the activity was very extensively destroyed by the process of aerosolization, sometimes by as much as 90 per cent. Did you titer the amount of active enzyme that actually went into the mouth and throat of the patient?

M. Segal: We did not do that because it had already been done. H. G. Reiser has made such a study and has shown that there is no significant change in the proteolytic action of dornase. Becker has studied the aerosolized dornase against pooled pus, and we have used his data in setting up our procedure.

I should like to stress another point. We feel that every effort should be made to provide a free airway in patients who will undergo extensive lung surgery. We like to do it with "medical" bronchoscopy rather than actual bronchoscopy: by the use of water-vapor therapy for two or three days, then with extensive dornase therapy for the day prior to surgery. Then, after surgery we are in favor of continuous administration of water-vapor therapy in a permanent hood-type of apparatus.

USE OF ENZYMES IN VETERINARY MEDICINE

By Charles W. Raker

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The proteolytic enzymes have been employed in veterinary medicine as adjuncts in the treatment of a variety of infectious and noninfectious disease processes. In early trials these enzymes were used in many conditions in which it was thought that they might be of value. The results of these early investigations were gratifying. Conditions that had previously been considered refractory or had been slow to respond to conventional therapy were found to respond more readily when enzymes were included in the therapeutic program. In inflammatory conditions the recovery phase was shortened, and better response to the antibiotic agents employed was noted.

In veterinary medicine the practitioner is frequently faced with the problem of balancing the prognosis and the cost of treatment against the economic value of the patient. By accelerating the recovery period, the proteolytic enzymes have aided the veterinary practitioner, the client, and, of course, the patient.

Investigations by Sherry *et al.*[1] showed that one of the principal components of purulent exudates was desoxyribonucleoprotein, and that the enzyme desoxyribonuclease had the capacity to degrade this protein and to reduce the viscosity of the exudate.

These enzymes, which are specific biochemical organic catalytic agents,[2] are derived from several sources. The scope of activity of the specific enzyme depends on its origin and nature.

Pancreatic Dornase

This enzyme, derived from bovine pancreas, breaks down desoxyribonucleic acid. McCarty[3] reported that the end product was composed of particles of relatively large size. This incomplete degradation probably decreases the quantity of the toxic substances absorbed into the system.

The action of pancreatic dornase is primarily one of liquefying mucopurulent exudates. Limited activity on fibrin and mucus is shown; this is probably the result of the presence of small amounts of trypsin and chymotrypsin.[4]

Pancreatic dornase has been used almost exclusively by local or topical application. It has been applied to suppurating wounds and has been packed in cavities, using gauze sponges saturated with 100,000 units or more of freshly prepared solution. In many instances antibiotics have been incorporated without apparently influencing the activity of the enzyme to any marked degree.

Drury[5] was of the opinion that pancreatic dornase was of value in the treatment of bovine mastitis. Bixler[6] reported favorably on his experiences with this enzyme in acute and chronic bovine mastitis, in abscesses, in frontal sinusitis in bovines following dehorning, and in otitis externa in the dog. In the case of mastitis, the proteolysis that resulted facilitated removal of the exudate via the teat and permitted more favorable response to the specific antibiotic or chemotherapeutic agents normally used in treatment. The enzyme did not appear to be harmful to the glandular tissue of the udder and, when applied in the treatment of wounds and abscesses, stimulated normal healing processes.

Kral[7] reported a new therapeutic approach for handling cases of primary sinusitis in equines. Instead of employing the usual technique of establishing one or more relatively large trephine openings into the sinus to permit drainage and allow for adequate treatment, a small opening through which a cannula could be introduced was made. A solution of pancreatic dornase or a mixture of streptokinase and streptodornase was then introduced to liquefy the exudate. This treatment was followed by local therapy with an antibiotic chosen on the basis of sensitivity tests conducted on cultures obtained from the exudate.

In addition, pancreatic dornase has been used successfully in treating cases of traumatic pericarditis in bovines.[8] In one instance, removal of the causative foreign body was accomplished by a rumenotomy. Following drainage of the pericardial sac by paracentesis and the introduction of pancreatic dornase and of antibiotics, marked clinical improvement was noted. Administration of the enzyme, in doses of 100,000 units combined with the antibiotics, was continued daily until no further exudate could be aspirated. Systemic antibiotic therapy was administered in conjunction with the local treatment and a complete recovery resulted. In those cases in which the removal of the offending foreign body cannot be accomplished, the condition of the animal can usually be improved sufficiently so that it can be sent to slaughter for edible purposes.

Scheel[9] has described a procedure employing pancreatic dornase as an aid in the treatment of infectious bovine rhinotracheitis. A combination of 100,000 units of pancreatic dornase in 100 cc. of diluent, 10 cc. of a penicillin-dihydrostreptomycin preparation, and 20 cc. of water or 50 per cent glucose solution was introduced into the trachea through a 16-gauge needle inserted between the tracheal rings. Observations indicated that the exudate was liquefied sufficiently to enable the animal to expectorate quite freely. While one treatment was usually sufficient, on occasion it was necessary to repeat the therapy at 24- to 48-hour intervals.

In view of Scheel's work in infectious bovine rhinotracheitis, we are conducting further investigations utilizing the principle of enzyme proteolysis in infections of the respiratory tract. In our current clinical investigations we are utilizing special equipment prepared for large animals to introduce the enzymes and antibiotics in the form of an aerosol combined with oxygen. The number of cases treated up to the present is too low to allow statistical evaluation. However, this method does show promise in the treatment of pneumonia, chronic pulmonary emphysema, and bronchitis. The treatment is well tolerated and apparently free from side reactions.

The respilator* used in these investigations was designed for large animals and has been described by Reed and Glasser.[10] Adequate aerosolization is effected with a sufficient flow of oxygen to render the application of the head mask comfortable. The aerosol employed contains Dornovac* combined with a broad-spectrum antibiotic or a combination of penicillin and dihydrostreptomycin. The mixture is contained in a 20-cc. volume and is administered for a period of 20 min. twice daily.

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* The respilator was developed by Professional Veterinary Service, Miami, Fla., and made available through the courtesy of Merck Sharp & Dohme, Division of Merck & Co., Inc., Philadelphia, Pa.

Trypsin

Trypsin, a purified crystalline enzyme derived from mammalian beef pancreas, has been used both locally and systemically in veterinary medicine as a proteolytic enzyme.[11] The systemic administration of trypsin has been restricted to intramuscular injection. Locally, it has been infused into the sinuses, the mammary gland, and the uterus, and applied as moist packs to suppurative surface wounds, ulcers, exuberant granulations, and fistulous tracts. Johnson and Ferguson[12] reported on the use of chymotrypsin in a number of clinical entities in animals. This enzyme, which exhibits both fibrinolytic and proteolytic activity, has been effective in the treatment of traumatic pericarditis, sinusitis, foot canker, ulcers, suppurative arthritis, osteomyelitis, and fistulous withers.

Because of the undesirable effects of intravenously administered trypsin on coagulation of the blood, the only parenteral route of administration used in animals has been the intramuscular. A preparation of trypsin in sesame oil designed for intramuscular injection has been reported to be effective in inflammatory conditions, such as cellulitis, edema, and lymphangitis in the horse.

Streptokinase and Streptodornase

This combination of enzymes, produced by a strain of streptococcus and available as Varidase†, is an effective fibrinolytic and proteolytic therapeutic agent. Varidase exhibits activity locally, as well as upon parenteral administration. Streptokinase activates fibrinolysis, causing dissolution of fibrinous exudates and of blood clots, and streptodornase acts directly to break down desoxyribonucleoprotein, thus lowering the viscosity of the purulent exudate.

The intramuscular administration of streptokinase-streptodornase in human patients has been shown to be effective in a variety of inflammatory and edematous conditions.[13] As an active fibrinolytic agent, streptokinase activates the reaction of plasminogen in human blood serum to form the active proteolytic enzyme plasmin, which is responsible for the fibrinolysis observed.[14]

As an anti-inflammatory agent, streptokinase accelerates the recovery period in acute inflammations. Fibrin is present in sizable amounts in inflammatory exudates and, according to Easterbrooks *et al.*,[14] its presence is nature's method of walling off the inflammatory process. At the same time this so called "limiting membrane" may prevent therapeutic agents from gaining access to the area and exerting their effects. Streptokinase-streptodornase, although it does not display any action upon normal tissues, appears to lyse this fibrin, attacking it from the periphery. It is also active centrally. Upon removal of this barrier, the chemotherapeutic agents are capable of reaching the site of the infection.

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* Merck Sharp & Dohme, Division of Merck & Co., Inc., Philadelphia, Pa.

† Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

In applying the above observations to animals, Easterbrooks *et al.*[11] reported that *in vitro* tests failed to demonstrate that streptokinase would activate plasminogen in bovine plasma to produce plasmin, and that only a percentage of the plasminogen in canine plasma was activated. Additional investigations revealed that, on adding human plasminogen to streptokinase, plasmin was formed, and that an apparently new but unidentified substance was also elaborated. This new substance, designated by the letter *X*, in the presence of streptokinase stimulates the activation of bovine plasminogen to plasmin. This reaction appears to occur in the plasma of all species of animals. The combination of these enzymes, consisting of streptokinase and streptodornase plus human plasminogen, should then assure activity in the plasma of all species of animals.

Toxicity studies have shown that five times the recommended therapeutic intramuscular dose, when given intravenously, has not resulted in any undesirable side effects in bovines. Similar results were obtained in dogs.

Streptokinase-streptodornase plus human plasminogen is being investigated as a therapeutic agent in a number of disease conditions in both large and small animals. Even if the condition under treatment is not of an infectious nature, a broad-spectrum antibiotic should always be administered concurrently because the fibrinolytic activity of the streptokinase may result in the release of an old encapsulated infection.

Riley[15, 16] has recently reported on the effects of streptokinase-streptodornase plus human plasminogen on a number of equine cases of a stubborn nature. Favorable results were obtained in cases of cellulitis, myositis, periostitis, septic arthritis, abscess, bone fistula and osteitis, supraspinous bursitis (fistulous withers), and infected wounds. The enzymes were administered by the intramuscular route in doses representing 100,000 units of streptokinase plus human plasminogen, accompanied by an adequate dose of tetracycline. Administration was continued for two or more days, depending upon the response shown.

Leventhal and Easterbrooks[17] observed gratifying results in the treatment of infectious pododermatitis in bovines. Several cases treated were considered terminal but, instead of resorting to claw amputation, they instituted therapy with streptokinase-streptodornase plus human plasminogen and antibiotics. In all of the cases treated in this manner surgical intervention was not necessary due to the marked improvement shown; reports on the condition of these cows months later indicated that the results were maintained. In treating this type of case it is recommended that the streptokinase-streptodornase (100,000 units streptokinase and 25,000 units streptodornase) plus human plasminogen and the antibiotic be administered once daily for two to four days.

Other conditions in the bovine, equine, canine, and feline species reported[18] to be amenable to therapy with streptokinase-streptodornase plus human plasminogen include: actinomycosis, bronchitis, hematoma, mastitis, otitis, pneumonia, dermatitis, and post-parturient edema of the udder.

In equines streptokinase-streptodornase plus human plasminogen has been administered in several cases of cellulitis involving the legs. In all cases treated the condition had been of long duration. Treatment was carried on for approximately seven days; observations and measurements of the legs did not reveal any reduction in size. However, one case had, in addition, a draining sinus tract from which a pure culture of a β-hemolytic streptococcus was obtained. On the fourth day of treatment the character of the exudate was noted to have changed to a more serous consistency and, following termination of the enzyme therapy, practically all exudation had ceased.

Local tissue irritation has been observed in several cases treated. The most disturbing reactions occurred when the streptokinase-streptodornase plus human plasminogen was injected into the anterior pectoral muscles. In one instance the leg on the side in which the injection was made became edematous, hot, and painful, and the horse became lame. No treatment was given for this edema, and within seventy-two hours the reaction commenced to subside. The preferred site for administration in the equine and bovine is in the neck muscles; the injection is given on alternate sides on each successive day of treatment. The preparation of streptokinase-streptodornase plus human plasminogen is reconstituted into solution with 20 cc. of sterile physiological saline and is administered by deep intramuscular technique, depositing not more than 10 cc. per site.

Miller *et al.*[18] reported that ten per cent of the human patients comprising their study showed a temperature rise. This same reaction has been observed in two equines to date. One patient, a four-year-old Standard bred horse, was referred to the hospital for treatment of lameness in one of the hind legs. A diagnosis of thrombosis of the external iliac artery was made and, although the condition was of four to six weeks' duration, it was decided to try enzyme therapy. On the day following the first dose, the temperature rose approximately one degree. Administration of the enzyme was continued and on the two succeeding days a continual rise was noted. Due to the monetary value of the patient no further streptokinase-streptodornase plus human plasminogen was given and, within twenty-four hours, the temperature had returned to normal. A recent report indicates that the condition of the thrombus is unchanged.

Another interesting observation was made on a cow referred for treatment of traumatic pericarditis. An attempt to recover the foreign body via a rumenotomy was unsuccessful. Thus, it seemed advisable to open and explore the pericardial sac in an attempt to recover the causative agent. Pericardial drainage was established by a resection of the sixth rib, approaching from the left side of the thorax. It was thereby possible to recover the foreign body. The cow was then placed on a program of streptokinase-streptodornase plus human plasminogen and antibiotics administered systemically but, due to the gradual deterioration in condition and the failure to respond to the treatment, she was eventually destroyed. The sutures used in closure of the abdominal wall following the rumenotomy had been removed on the ninth day. Healing had appeared to be normal but, on the following day, the wound had reopened. Although observations made at this time did not reveal the presence of infection, there was a complete lack of union of the skin and muscles. It was felt that the enzymes played a part in the apparent failure of this clean surgical wound to heal in the normal manner.

The anti-inflammatory properties of streptokinase-streptodornase plus human plasminogen have been demonstrated in a number of cases. One example is a bovine that developed, following a hysterotomy, an extensive edema with accumulation of serum in the abdominal wall at the site of closure. Two doses of streptokinase-streptodornase plus human plasminogen and a broad-spectrum antibiotic were given on consecutive days. Improvement was noted on the second day, and recovery was complete by the fourth day.

Discussion and Conclusion

Proteolytic enzymes have been demonstrated to be effective therapeutic aids in the treatment of a variety of inflammatory and edematous conditions in animals. Pancreatic dornase is effective when placed in direct contact with the inflammatory process. Its action is proteolytic, breaking down viscid purulent exudates into exudates having a fluid consistency and small particle size. Normal healing of wounds is accelerated and exuberant granulations depressed. Pancreatic dornase is not effective upon parenteral administration.

Trypsin and chymotrypsin, two closely related enzymes, are active both by local and parenteral administration. Veterinary use of these agents has been confined to intramuscular and local application. In addition to their ability to break down purulent exudates, they possess fibrinolytic activity, which renders them of value in a wider range of cases.

Varidase is a combination of streptokinase and streptodornase. The addition of human plasminogen is necessary to activate the plasminogen in the plasma of animals to produce the active fibrinolytic enzyme plasmin. Varidase plus human plasminogen appears to be an effective proteolytic enzyme for parenteral administration in animals. A broad-spectrum antibiotic should be administered in conjunction with these enzymes to control the release of any evident or cryptic infection by fibrinolysis of the so-called "limiting membrane." Doses of streptokinase (100,000 units), streptodornase (25,000 units), plus sufficient human plasminogen to activate the formation of plasmin, are recommended for equines and bovines. Correspondingly smaller doses are recommended for the smaller species of animals.

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THE PRINCIPLES INVOLVED IN THE TOPICAL USE OF STREPTOKINASE-STREPTODORNASE

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In the study of the enzymes elaborated by hemolytic streptococci, their actions as observed under experimental conditions have been the essential basis for the development of their clinical use. In pursuing this method it has been the aim of my associates and myself to correlate the principles involved in the reactions, both *in vitro* and *in vivo*, with the objectives sought in the therapeutic application of these enzymes. It has been, and continues to be, our endeavor wherever possible to utilize both qualitative and quantitative methods of measurement, as opposed to empirical clinical appraisal. The subject is by no means simple nor completely studied. New findings continue to emerge. Some of them help to clarify and others tend, at least temporarily, to confuse the orderly interpretation of both experimental and clinical results. In certain areas of disease, application of streptokinase-streptodornase (SK-SD) is regularly effective, and the therapeutic results are reproducible from patient to patient. In other instances the results have not been as satisfactory as theoretically might be expected. These latter circumstances have always excited our interest, and an understanding of the causes of failure has often added to our knowledge. For example, such a failure may contribute to the recognition of disease not previously correctly diagnosed. In still other instances, favorable clinical results have somewhat exceeded expectation. Such experiences have convinced us that between these enzymatic reagents and tissues there are in operation mechanisms that have broader biological implications than the simple degradation of specific substrates.

I shall not at this time attempt to present a documented protocol of the points I have briefly outlined. However, when all the mechanisms of tissue behavior as influenced by enzymatic application are understood and can be measured and applied, further advances in the practical features of SK-SD therapy may be anticipated.

I should like to call attention to certain biological aspects of the streptococcal enzymatic phenomena. The two enzymes, streptokinase and streptodornase, that hemolytic streptococci have presented to us for study are dissimilar in every respect. Both their production and their mode of action are entirely independent of each other even though they are produced simultaneously by the same microorganism. However, they do have one common denominator that is of considerable interest and deserving of attention. Fibrin, which the SK system attacks, and desoxyribonucleoprotein (DNA protein), which SD attacks, both belong to the same chemical group of substances designated as fibrous insoluble proteins. Although these proteins are entirely different in chemical structure, they have comparable physical properties of coarseness, stringiness, and stickiness. Consequently, when either one or both of them appear as constituents of pathological processes they pose a particularly difficult problem for the body to meet through its own processes of repair.

Fibrin as such does not exist in tissues under normal conditions. Transformed from its precursor fibrinogen, it emerges with greatest frequency under conditions of disease or trauma and, not infrequently, it is a significant deterrent to recovery. Because of the importance of the prime function of fibrin in arresting hemorrhage by creating a mechanical barrier, nature has endowed the substance with a tenacious insoluble quality that is not subject to quick or casual change under normal conditions. Treatment has formerly been by surgical procedures. Without surgical intervention the offending layers of fibrin may be eradicated only by the enzymatic action of the body's own proteinases and or fibrinolysins. The amount of these endogenous liquefiers is, as a rule, quite limited, and they do not collect regularly in high concentrations locally where blood has been extravasated. When the pathological depositions of fibrin are extensive the natural enzymatic mechanisms of the body may be grossly insufficient, with the result that adhesions form and functioning tissues are replaced by inert fibrous material, representing permanent damage.

Similarly, DNA protein is thick, viscous, and stringy, and it gives to purulent exudate its tenacious gummy quality. The nucleoproteins, which are among the largest of all proteins, are also referred to as insoluble fibrous proteins, as contrasted with the so-called corpuscular or globular proteins (such as albumin, globulin, and a large number of others), which are readily soluble. In this connection it is interesting to point out that of all the proteins that make up the tissues of the body, only fibrin, DNA protein, and some of the connective tissue proteins such as collagen, reticulin, and elastin belong in the category of the insoluble fibrous type. DNA protein, for example, has a molecular weight of 1,000,000 to 2,000,000, in contrast to hemoglobin, which has a molecular weight of 68,000. The removal of pathologically occurring DNA protein from patients is by surgical means or, when spontaneous rupture does not occur, by the body's own endogenous dornase, which is present in the circulating blood in very small quantities (0.05 to 0.1 unit per ml.). The endogenous enzyme has not been found to collect in particularly high concentration locally. As in the case of fibrin depositions, removal of DNA protein by endogenous enzymatic action, subsequent absorption, and ultimate excretion requires protracted periods.

Because of the special physical and chemical characteristics of the two proteins discussed above, it is of considerable interest to speculate how one single group of bacteria, β-hemolytic streptococci, in its evolution and development acquired, perhaps through eons of time, these two separate and independent enzyme systems that specifically attack the two proteins mentioned.

As a generalization it may be stated that the properties of any viable cell serve one of three purposes: self protection, nutrition, or reproduction. Which of these purposes do SK and SD fulfill for streptococci? Because of the conspicuous identification of hemolytic streptococci as human pathogens it is most natural to assume that the liquefying properties acquired by hemolytic streptococci promote the capacity of the microorganisms to spread through the body and continue to find wider areas in which better to obtain nutritive materials and to multiply. In so doing they cause diseases and may be even fatal to the host. This human equation is an interesting one. The β-hemolytic streptococci most commonly found to possess the fibrinolytic property belong to Group A, which is distinguished as containing the most common human pathogens Furthermore, leaving aside the details of the mechanism of SK activity, it is a fact that in the simple testing of a broth culture of a strain of fibrinolytic streptococci, the fibrin clot of human blood is much more susceptible to lysis than that of animal bloods. It is suggestive, therefore, that in the evolution both of man and of streptococci a period of long and enduring contact has influenced the emergence of the streptococcal properties under discussion.

According to one classification of bacterial enzymes, the fibrinolytic property belongs to the group designated *constitutive enzymes*, which do not require the presence of substrate for the production of the enzyme, as opposed to *adaptive* enzymes; the latter are formed only in the presence of the specific substrate. In earlier studies I was never able to demonstrate that the addition of fibrinogen or fibrin to culture media devoid of other protein constituents enhanced the growth or fibrinolytic activity of the strains. I have not made similar cultural studies with respect to additions of DNA proteins.

From this brief description it is apparent that these two proteins pose unique difficulties to the processes of recovery. It is proposed, therefore, to overcome these difficulties by introducing locally the effective enzymes, SK and SD, in high concentration an effect that the body by its own mechanism is not able to achieve and through the speed of their action to alter local conditions in a short period of time.

In the brief review just given, I have attempted to outline the essential biological factors from which this total study of streptococcal enzymatic therapy has emerged.

In the field of practical therapy, publications resulting from our studies and those of other investigators* have described the types of diseases in which SK and SD have proved to be highly advantageous therapeutic reagents when employed locally at the site of disease.[1-3]

It is unnecessary to describe the results again, but they may be outlined as follows:

Direct Effects Based on Enzymatic Specificity

(1) Effective specificity of the SK fibrinolytic system in cases of hemothorax and other instances of large hematomata.

(2) Effective specificity of SD in combination with SK in diverse diseases

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* For a comprehensive review of recent articles on the clinical use of streptokinase-streptodornase (Varidase) see the bibliography contained in "Recent Advances in Varidase® Therapy," distributed by Lederle Laboratories Division, American Cyanamid Co., 30 Rockefeller Plaza, New York 20, N. Y.

involving local purulent infections in which DNA protein constitutes a large portion of the exudative material.

(3) Effect of SD in local abscesses made up almost exclusively of DNA protein.

In the above types of pathology, the transformation of the stringy viscous insoluble protein materials into limpid solutions has permitted rapid and effective aspiration or drainage.

Indirect Effects

(1) Eradication of the offending bacteria by permitting the entrance of leukocytes, of serum antibodies, and of circulating antibiotics into the area of infection. The so-called walling-off process, by which infections are presumed to be localized, in many instances does not protect the tissues from the bacteria but it does, on the other hand, protect the bacteria from the effective antibacterial forces of the body.

(2) Wound healing. In many instances healing of large infected areas of soft tissue has proceeded at an unusually rapid rate after the debridement effect of intensive topical application of solutions of SK-SD has become evident. The results have suggested that the breakdown products of enzymatic degradation may possibly have served locally as an enriched culture medium which promoted the rapid regeneration of the local cellular structure. Studies of this aspect are in progress.

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THE EVALUATION OF ANTI-INFLAMMATORY DRUGS IN SURGICAL LESIONS*

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As the title of this report indicates, several enzymatic compounds with anti-inflammatory properties, rather than one, were studied. This study was initiated in an attempt to find a parenteral proteolytic enzyme to debride patients with burn eschar. In this approach to the treatment of burns there are numerous problems connected with the deposition of a foreign protein substance in the tissues. The majority of the proteolytic enzymes demonstrating efficient local activity as debriding agents on denatured human skin collagen have been found to be extremely toxic *in vivo*.

However, the parenteral use of streptokinase-streptodornase, trypsin, and the corticoids is relatively safe. Although these substances were relatively inefficient in the treatment of burn eschar, they exhibited a number of interesting and beneficial activities in patients with inflammatory lesions. Certain proteolytic enzymes were capable of rapidly reducing the local edema, erythema, heat, and pain found in connection with acute inflammation and infection. The exact mechanism by means of which this occurred is not known at present, but it is believed that the enzymes lyse plugs of fibrin in the lymphatics and capillaries about the inflammatory lesion.

Methods and Materials

The materials used and compared in this study were: streptokinase-streptodornase (Varidase), trypsin (Tryptar), cortisone (Cortone), hydrocortisone (Hydrocortone), and phenylbutazone (Butazolidin). The known chemical and physiological properties of these materials have been adequately covered in previous reports.[1-6] The materials are available as sterile lyophilized powders that are easily put into solution by the use of buffered diluents or normal saline. In a number of instances 0.5 per cent procaine solution was added to the mixture to reduce pain at the injection site. None of the materials were given intravenously; phenylbutazone was given orally. Dosages and intervals between administrations were determined by empirical testing. In all instances of infected lesions treated by these agents, a broad-spectrum antibiotic was administered during the course of therapy.

Clinical experiences were obtained on the surgical wards of The St. Vincent's Hospital of the City of New York. The lesions studied consisted of abscesses, abscesses with cellulitis, cellulitis, phlebitis, wound infections, bursitis, myofascitis, and hematomata. For most patients the following

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* This study was supported in part by the Department of Medical Research and Development of the United States Army, Washington, D. C. and by the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

determinations were made daily: prothrombin time, blood count, sedimentation rate, and temperature. Blood cultures, taken at indicated intervals, and frequent urinalysis and blood nitrogen studies completed the laboratory survey for toxic manifestations.

Results

These anti-inflammatory agents have been studied in two hundred or more patients. To enable us to compare the qualitative responses, we drew upon our large background of studies from the Vascular and Surgical Services of the hospital made with sodium adenosine-5-monophosphate (My-B-Den), the anticoagulants, and the antibiotics. In these studies the response to the therapeutic agent had been compared with that of untreated patients or of patients in whom other recognized regimens had been utilized.

Streptokinase-streptodornase (*Varidase*). The majority of patients in this study were treated with streptokinase-streptodornase (TABLE 1). Prior to the intramuscular use of this agent we had had four years' experience with its local application. For intramuscular use the enzyme was diluted with sterile normal saline prior to injection. Early in the study we decided to add 0.5 ml. of a 1 per cent procaine solution to the enzyme to decrease the local pain associated with injection. Various dosages and intervals between dosages were studied; these dosages ranged from 5000 units twice daily to 20,000 units every 8 hours. One group was given 20,000 units once a day for short-term courses. There appeared to be a correlation between dosage and toxic manifestations. Thus, in most instances where a 20 per cent increase in prothrombin time occurred, the accumulated dosage was approximately 50,000 to 60,000 units. In instances where Arthus phenomena occurred, the accumulated dosage exceeded 100,000 units. On the other hand, in many cases febrile reactions occurred following the first injection of 5000 units.

Streptokinase-streptodornase gave uniformly excellent results in patients with cellulitis. Therapy was continued for approximately 48 hours or until 20,000 to 40,000 units had been administered.

TABLE 1
STREPTOKINASE-STREPTODORNASE

	No. of patients	Average duration of lesion pretreatment	Duration of treatment	Patients with toxic reaction	Results
Cellulitis	10	72 hours	2 days	2	Excellent
Abscess	10	39 hours	6 days	9	Fair
Abscess with cellulitis	8	48 hours	8 days	8	Good
Phlebitis	12	30 hours	3 days	3	Good
Wound infection	10	29 hours	3 days	4	Good
Burns	10	4 hours	10 days	10	No effect
Lumbosacral strain	4	34 hours	6 days	3	Good

Abscesses with spreading cellulitis (FIGURE 1) responded more dramatically than abscesses alone. Only a combination of antibiotics and enzymes meets the requirements for therapy. In one case where no antibiotic was given, the abscess required 10 days of enzyme treatment before resolution became apparent. This patient did not develop bacteremia because the abscess was well walled off, but there is no justification for considering the enzyme as a bactericidal agent and, since we assume that the enzyme is lysing the natural

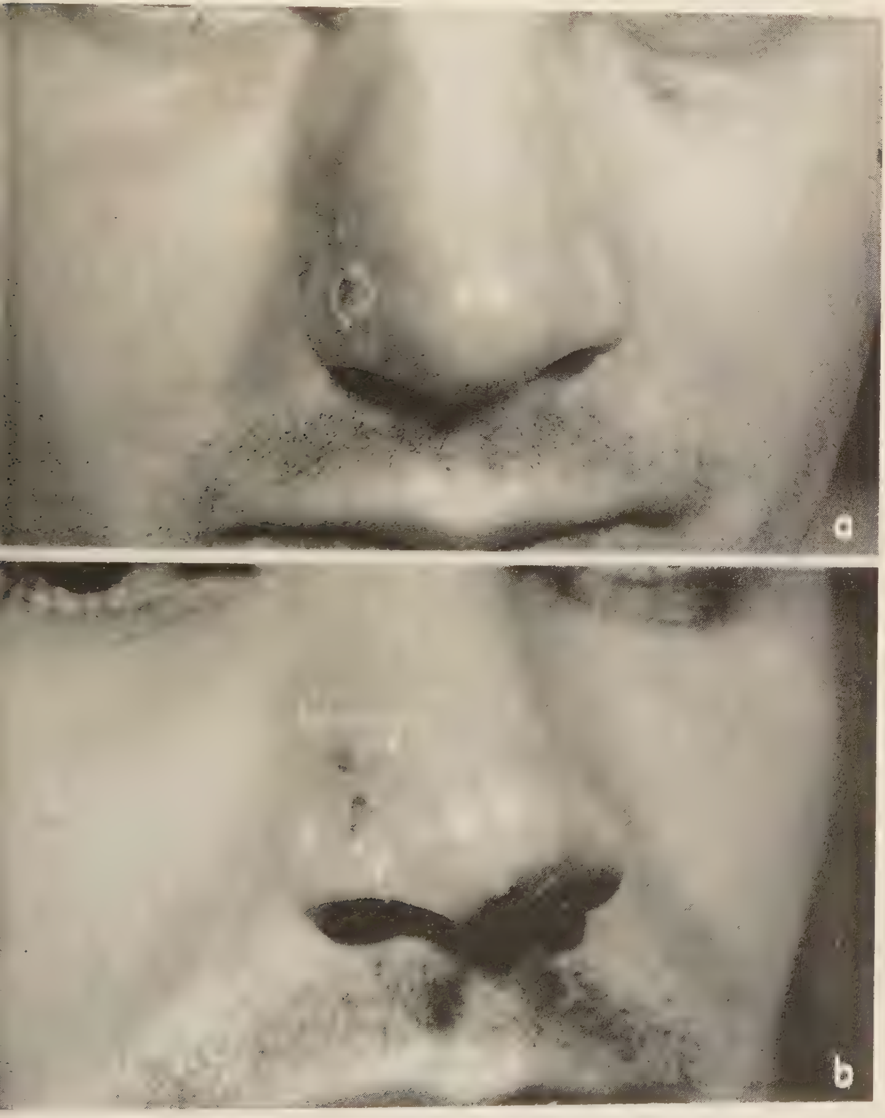

FIGURE 1. (a) Abscess with cellulitis of the nose; (b) after 39 hours of combined parenteral streptokinase-streptodornase and antibiotic therapy.

fibrin barriers in the capillaries and lymphatics, it is exceedingly dangerous to withhold antibiotics. The use of anti-inflammatory enzymes must be considered only as an important adjuvant to antibiotic therapy. We say "important" because we feel that any material increasing the speed of healing will tend to inhibit the development of organisms resistant to the antibiotic.

Acute phlebitis, with or without a concomitant ulcer, responded well, as far as edema and inflammation were concerned, after 48 to 72 hours on 5000 units every 12 hours (FIGURES 2 and 3). Ambulation was permitted in many patients and it had no effect on the therapeutic response. Patients with chronic phlebitis usually required a longer course of therapy to bring about subsidence of edema. The usual duration of therapy was 8 days with accumulated dosages of 100,000 units.

Patients with wound infections responded similarly to those with cellulitis lesions.

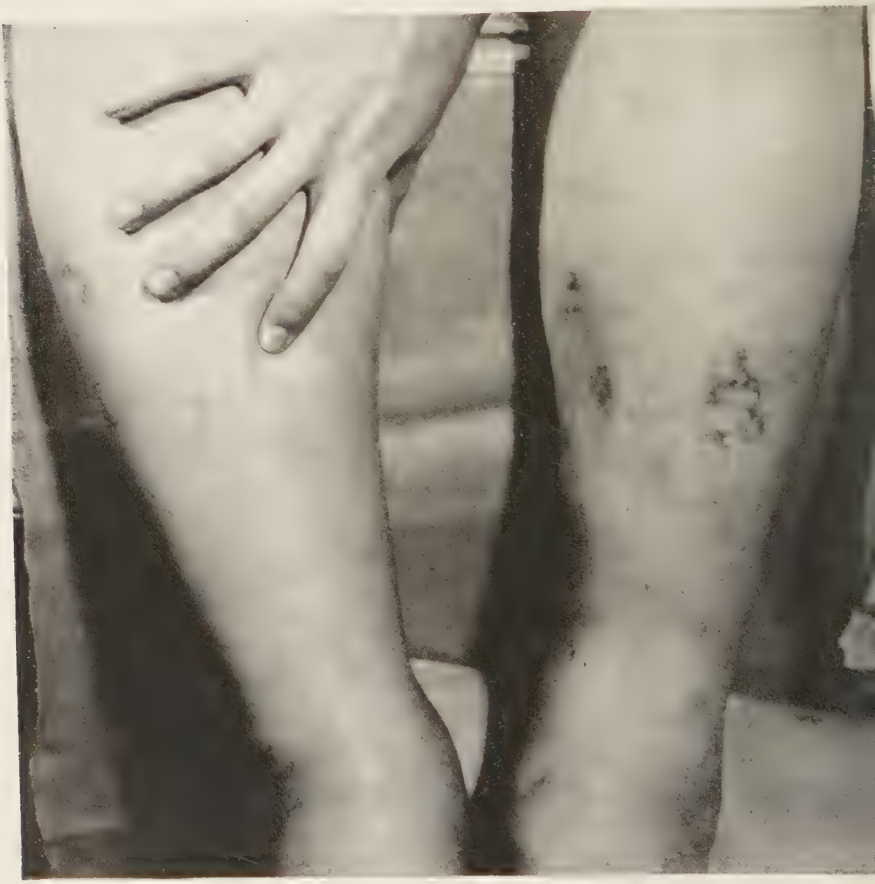

FIGURE 2. Bilateral peripheral edema secondary to phlebitis.

The patients with burns were studied from three aspects: (1) the effect of mobilization of fluid into the burned area; (2) the effect on the inflammatory reaction; and (3) the effect on debridement of the eschar. Although the results were in all instances difficult to demonstrate, it was evident that in no instance was edema prevented or caused to reabsorb faster than anticipated. In one case where the prothrombin time was elevated, a hemorrhagic diathesis into the blister fluid occurred on the fourth day. There was no effect on the erythema of the first-degree burn or on the inflammation about second- or third-degree lesions. In no instance did the eschar separate earlier than expected. In a few hand burns, we did feel that joint motion became freer and less painful at an earlier time than we had expected, but this observation is only a preliminary one (FIGURE 4).

Also studied were 4 patients with severe lumbosacral strain, with its concomitant edema and myositis. In 3 patients a definite alleviation of pain and improvement in motion were noted in 3 or 4 days of therapy on a dosage

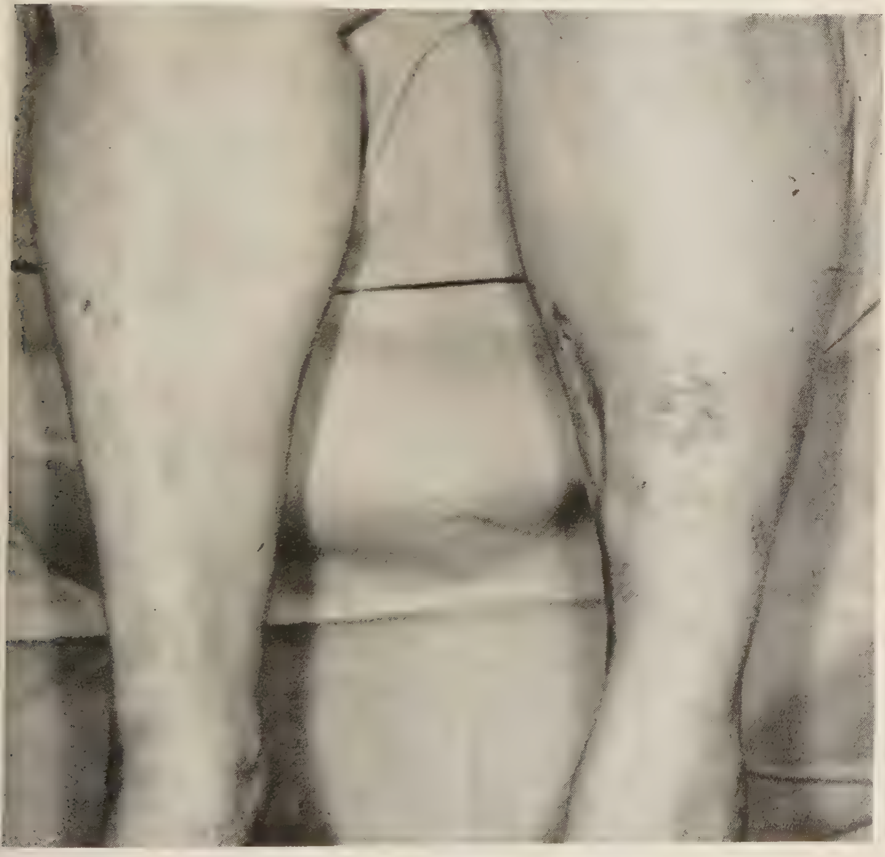

FIGURE 3. Follow-up photograph of patient shown in FIGURE 2, taken 56 hours after combined parenteral streptokinase-streptodornase and antibiotic therapy.

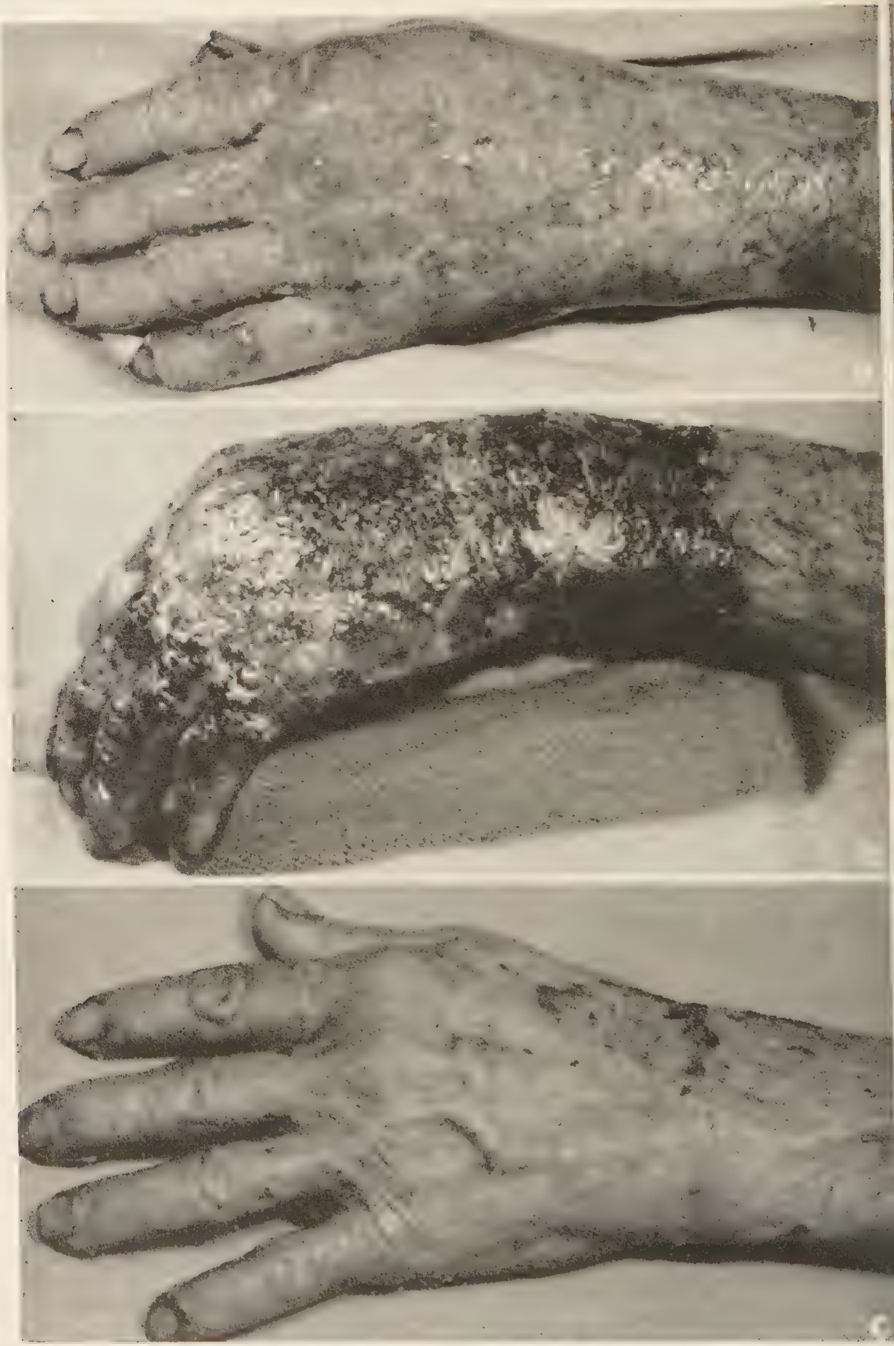

FIGURE 4. (a) Severe second-degree thermal burn of the hand 48 hours after injury. The patient was receiving parenteral streptokinase-streptodornase and antibiotics. (b

TABLE 2
TRYPSIN

	No. of patients	Average duration of lesion pretreatment	Duration of treatment	Patients with toxic reaction	Results
Cellulitis	5	10 hours	2 days	0	Excellent
Abscess	5	42 hours	6 days	2	Fair
Abscess with cellulitis	5	47 hours	7 days	3	Good
Phlebitis	3	30 hours	3 days	2	Good
Wound infection	4	24 hours	3 days	0	Good
Burns	3	4 hours	10 days	2	No effect
Bursitis	10	4 days	6 days	4	Fair

schedule of 5000 units every 8 hours. The fourth case had a severe allergic reaction to the drug, requiring discontinuation of therapy.

The patients with hematoma were difficult to assay. The hematomata following bruises responded well, but lesions secondary to fracture were more complicated. We feel that the edema secondary to fracture was quickly controlled, but that further hemorrhage continued from the fracture site. This group will require more study before we care to recommend acceptance or rejection of enzyme therapy.

Toxic manifestations will be discussed in the following section.

Trypsin. As in the previous group, trypsin, as Tryptar, was studied initially in its application to patients with burns (TABLE 2).

The results with this material in the various clinical entities described above were similar to those with streptokinase-streptodornase (FIGURES 5 and 6). The intramuscular dosage schedule varied from 5 mg. to 15 mg. twice daily. The material was dissolved in Sorenson's buffer containing 0.5 cc. of 1 per cent procaine solution.

The major differences in the two materials were related to their toxic manifestations. Both materials may provoke a pyrogenic response, with temperatures rising from 1° to 5° F. above normal or above the pretreatment hyperexia. Studies were carried out with blood cultures to learn whether this reaction might be secondary to bacteremia. No correlation has been found. The febrile reaction is not serious if dehydration is prevented. Although the usual antipyretic drugs may be given, they have little real effect. The hyperexia usually subsides spontaneously. With both drugs, painful injections are usual and the addition of a small amount of procaine, without mixing, into the enzyme solution drawn into the syringe just prior to injection has been helpful. In most patients given prolonged courses of

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After 7 days of therapy no changes were noted in the usual course of therapy without parenteral streptokinase-streptodornase. (c) Thirteen days after injury the patient had full mobility of his hand. The patient received combined parenteral streptokinase-streptodornase and antibiotic therapy for 10 days and local application of streptokinase-streptodornase for 3 days.

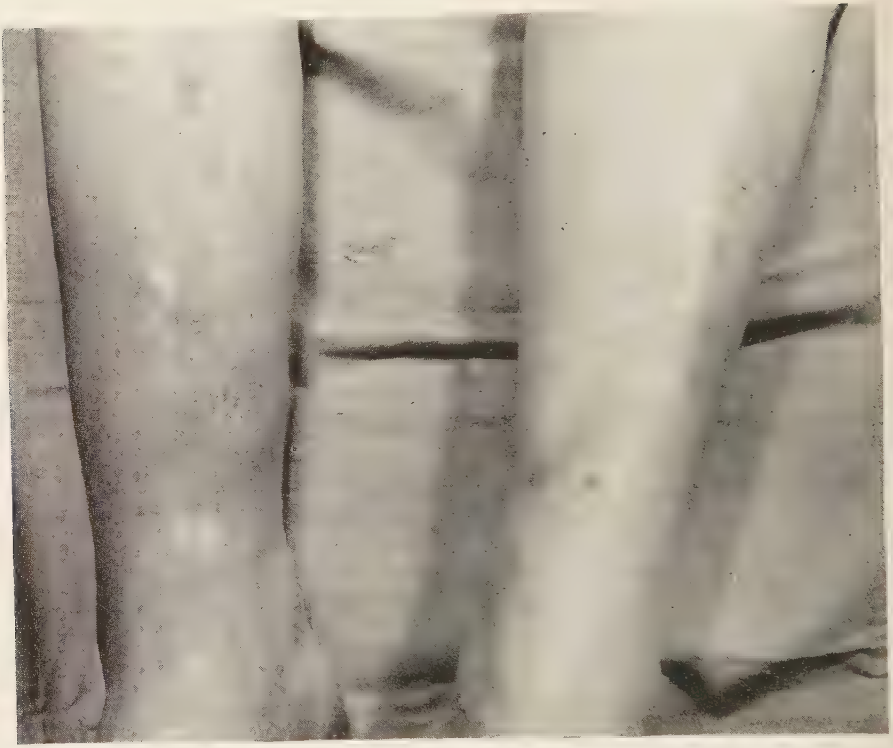

FIGURE 5. Patient with phlebitis and cellulitis of the right leg, approximately 72 hours after onset.

streptokinase-streptodornase, a significant rise of 20 to 30 per cent in the prothrombin time has occurred. In two instances the reaction occurred 48 hours after cessation of the drug. Usually the prothrombin time promptly returns to normal on cessation of therapy. There have been no instances of a thrombocytopenia or pancytopenia developing in conjunction with the elevated prothrombin time. We have not seen this reaction occur with trypsin, nor have we noted any clotting phenomena with the use of this material.

The most serious untoward reaction we have encountered has been the Arthus phenomenon (FIGURE 7). The initial reaction follows an intramuscular injection after a total of at least 100,000 units of the drug has been given over a 4- to 6-day period. This reaction consists of pain, edema, and erythema about the injection site. If a subsequent injection is given, the local reaction at the injection site increases markedly, and other injection sites demonstrate a similar inflammation. This reaction may resolve spontaneously in 48 to 96 hours, but during its course it is a frightening experience. We have found that 300 mg. of phenylbutazone (Butazolidin) every 8 hours will cause a more rapid subsidence of the signs and symptoms. It is our policy at present to limit the use of intramuscular streptokinase-streptodornase to 100,000 units total dosage and to discontinue the drug after 3 to 4 days of treatment. In patients in whom this reaction occurred, the intradermal injection of 50 units of streptokinase-streptodornase into the forearm 18 months after the first treatment resulted in a local reaction and tenderness over the previous gluteal sites of injection. In most patients developing this reaction, a careful clinical history demonstrated that they had had numerous upper respiratory infections, some of which were presumed to be streptococcal in origin. In a number of patients no such history was obtained. If the patient's history reveals past streptococcal infections, we now utilize one of the other anti-inflammatory substances. To date 5 patients have had severe reactions to streptokinase-streptodornase and 8 have had minor reactions of this type.

In the group treated with trypsin we have seen only 3 examples of this allergic manifestation. Again these patients were on prolonged courses of therapy with short intervals between injections. The reaction occurred after 4 days of therapy, with a total dosage of approximately 120 mg. or more. The dosage schedule was in each case every 8 hours. Similarly, with intramuscular trypsin therapy we have now limited injections to every 12 hours

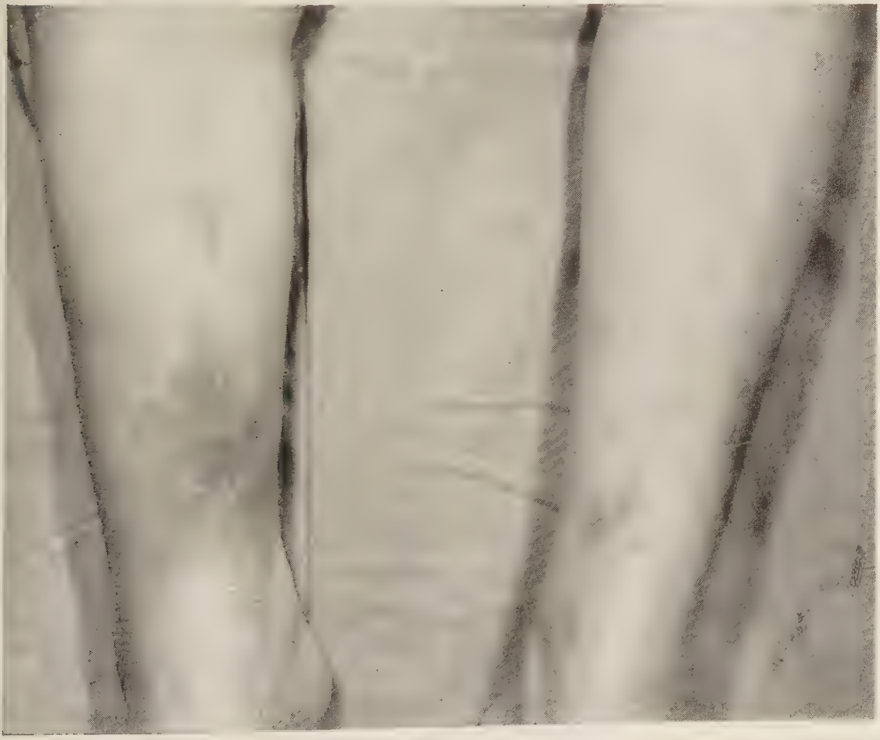

FIGURE 6. Patient shown in FIGURE 5. Resolution was noted after 72 hours of treatment with parenteral trypsin and antibiotics combined.

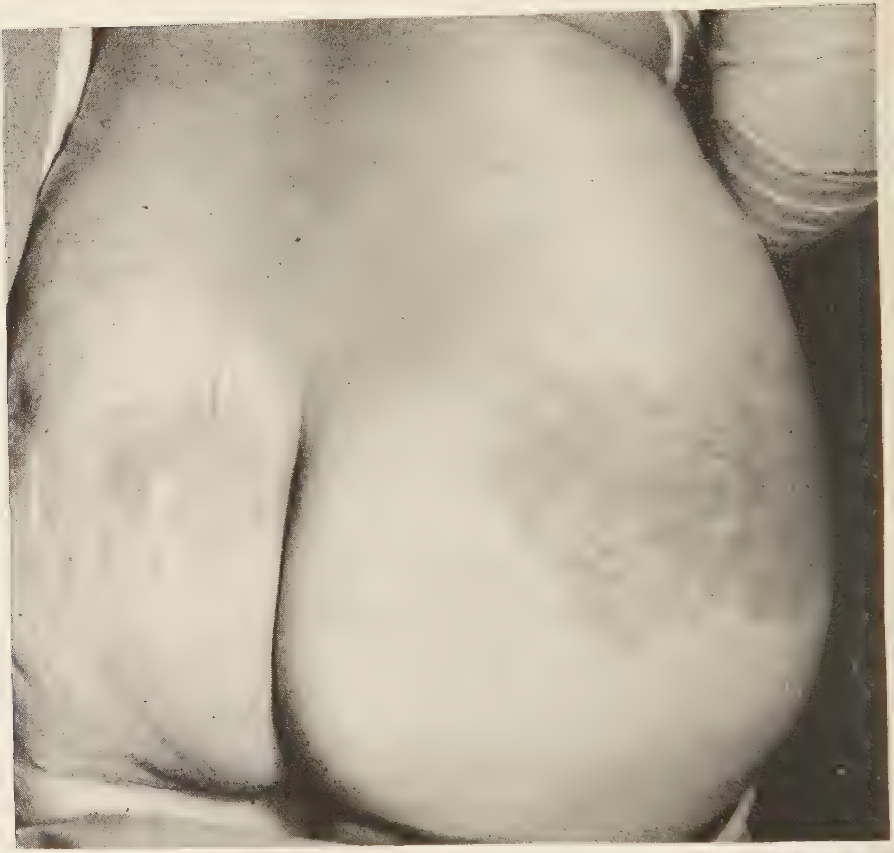

FIGURE 7. The last injection of streptokinase-streptodornase into the right buttock produced large erythematous lesions and a concomitant lesion over the site on the left buttock that had been used for previous injections. The reaction became worse, but no photographs were taken.

and the total dosage to 100 mg. Phenylbutazone therapy has been helpful in the patients with allergic reactions.

We did not employ trypsin in fracture patients.

Cortisone, hydrocortisone, and phenylbutazone. While these materials are not proteolytic enzymes, they are considered in this report for their anti-inflammatory activities (TABLES 3 and 4). Cortisone (Cortone) was given orally in 25 mg. doses every 8 to 12 hours for 2- to 4-day periods. Hydrocortisone, as Mercortin, was given intramuscularly in doses of 5 mg. every 8 to 12 hours. Phenylbutazone (Butazolidin) was given orally. The initial dose was 600 mg., followed by an 8-hour schedule of 300 mg. for 3 to 5 days. Prior to therapy the patient's history was investigated for gastric, renal, liver, or cardiac pathology.

In all cases a routine chest X ray was taken prior to therapy. If hyperpyrexia developed during the therapy the drug was discontinued and a

TABLE 3
CORTISONE AND HYDROCORTISONE

	No. of patients	Average duration of lesion pretreatment	Duration of treatment	Patients with toxic reaction	Results
Cellulitis	7	12 hours	2 days	0	Excellent
Abscess	6	40 hours	4 days	0	Fair
Abscess with cellulitis	5	38 hours	4 days	0	Good
Phlebitis	3	30 hours	3 days	1	Good
Wound infection	3	26 hours	3 days	0	Good
Burns	5	8 hours	6 days	3	No effect
Bursitis	15	3 days	6 days	0	Good

TABLE 4
PHENYLBUTAZONE

	No. of patients	Average duration of lesion pretreatment	Duration of treatment	Patients with toxic reaction	Results
Cellulitis	8	12 hours	2 days	0	Excellent
Abscess	7	42 hours	4 days	0	Fair
Abscess with cellulitis	9	37 hours	4 days	0	Good
Phlebitis	4	28 hours	3 days	0	Good
Wound infection	4	24 hours	3 days	0	Good
Burns	5	6 hours	5 days	0	No effect
Bursitis	15	3 days	5 days	0	Good
Lumbosacral strain	4	2 days	3 days	0	Good

second chest X ray was taken. These precautions followed from a lesson taught us by a patient with extensive burns who was given 130 mg. of cortisone over a 3-day period. His initial chest plate was negative, but on the third day, when his temperature spiked to 105° F., a second chest X ray revealed bilateral miliary tuberculosis.

These three drugs have demonstrated anti-inflammatory activities similar to the proteolytic enzymes already discussed. The major differences are the following:

They do not have any appreciable effect on hematomata or on suppurative wounds. In the carefully selected patient there is marked reduction in the number of untoward reactions. In a corticoid-dependent arthritic patient on hydrocortisone (Hydrocortone) with phlebitis of the left leg secondary to a cutdown, a pulmonary infarct developed after 12 days of therapy. Other reactions consisted of epigastric distress in the burn patients treated with cortisone. No gastrointestinal ulcerations were observed.

The group of patients treated with phenylbutazone was remarkable in that no untoward reactions were noted.

Discussion

The demonstration of the anti-inflammatory properties of these five drugs gives the physician an important adjuvant to his usual therapeutic regimen. These agents must be handled, of course, with considerable caution and an understanding of what they should be expected to achieve. The precautions presented in this report developed from our extensive survey to determine the most effective concentrations and dosage schedules. We do not wish to leave the impression that severe allergic reactions are inevitable or even so common as to preclude the use of the enzymatic agents. The agents will usually accomplish the desired therapeutic effect in total concentrations well under those that have produced serious reactions. In a few patients where short courses of therapy were indicated no allergic reactions occurred. However, our study indicates that the physician must not become negligent when using such powerful materials.

Conclusion

The drugs listed in this report, when employed in combination with the proper antibacterial agents, have ably demonstrated their ability rapidly to reduce the inflammation following injury or infection. Their use has measurably shortened the confinement of the patient to the hospital, with attendant economic savings.

Certain untoward reactions may occur with all five drugs. The more serious allergic reactions resulted from prolonged courses of treatment with large dosages of the proteolytic enzymes.

The mechanisms of action are yet to be explained, but in the meantime a most useful tool has been given to the physician confronted with inflammatory lesions.

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THE ANTI-INFLAMMATORY EFFECT OF PARENTERALLY ADMINISTERED PROTEASES

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In Walpole's classic tale the three Princes of Serendip were always discovering things they did not seek. In like fashion my own group set out to confirm and extend the observations of Johnson and Tillett[1] on enzymatic dissolution of clots; very unexpectedly, we discovered the anti-inflammatory properties of parenterally administered proteases. I should like first to review some of our early studies.

Intravenous administration of trypsin in dogs was found to cause an initial fall in clotting proteins, followed by a secondary rise in plasma antithrombin.[2] The antithrombic and fibrinogenolytic effects of intravenously given trypsin suggested that parenteral trypsin might alter the fibrinous structure of blood clots. Accordingly, thrombi were produced in marginal ear veins of 40 rabbits by injecting 0.5 cc. sodium sotradechol.[3] The end point used was cessation of blood flow on cut section. Three hours later 10 rabbits received, intravenously, 30 mg. trypsin; 10 rabbits received 30 mg. chymotrypsin; 10 rabbits received 50,000 units streptokinase every 4 hours for 5 doses; and the remaining 10 rabbits received saline by slow venous drip. At this dosage, the thrombi appeared to be relatively unchanged (FIGURE 1), but striking reversal of perivascular and interstitial inflammation had occurred. Cut sections of the ears of control rabbits failed to bleed and became infarcted. In enzyme-treated rabbits, despite the persistence of massive thrombi in major vessels, the thrombosed ears did not become infarcted and bled on cut section. In a previous study we had interpreted restoration of blood flow as an end point specific for thrombolysis. Histological observations in this study, however, suggested that removal of extravascular inflammatory barriers to flow of fluid (blood, lymph, tissue juices) in acutely injured tissue was somehow related to the reversal of inflammation and to the preservation of tissue viability in the enzyme-treated group. In short, by restoring and maintaining blood flow, metabolic continuity with adjacent healthy tissue was achieved. The acutely injured, inflamed tissue was no longer biologically isolated despite persistence of intravascular thrombi.

Animal and clinical studies show that parenterally administered proteases inhibit or reverse several phenomena associated with inflammation, each of which constitutes a mechanical barrier to blood flow, but on an extravascular level.

(1) *Edema.* The most consistent response to parenteral protease therapy is reduction in inflammatory edema. Martin[4] showed reduction of egg-white edema in rats following parenteral trypsin. Adamkiewicz[5] confirmed and extended this work. Cohen *et al.*[6] demonstrated inhibition of dextran edema

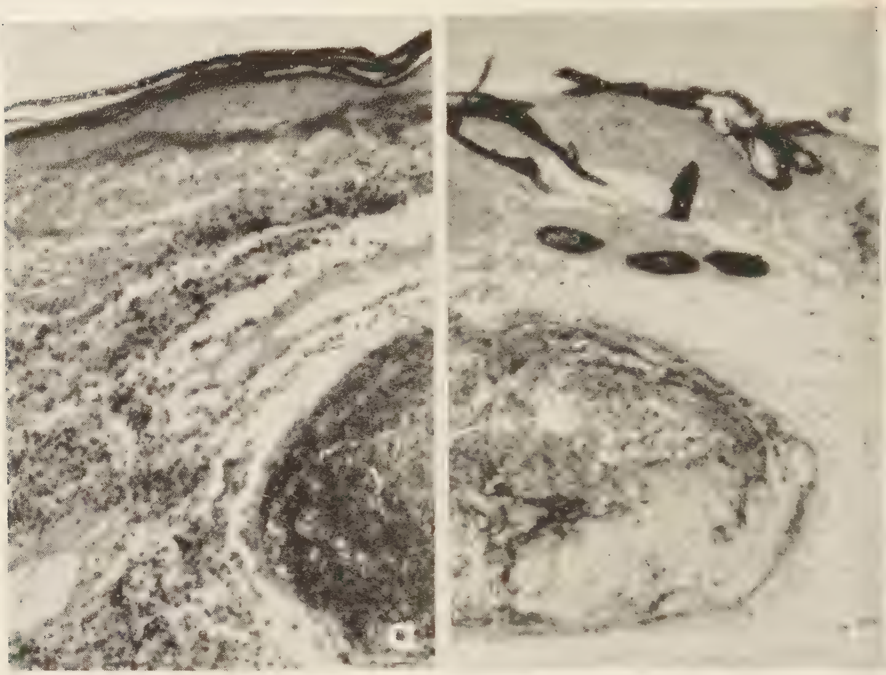

FIGURE 1. Morrhuate-induced massive thrombosis and interstitial edema. Rabbit ear. (a) Control. (b) Following chymotrypsin therapy there is removal of the inflammatory infiltrate and edema; the thrombus persists (*Surgery*, vol. 39, p. 426, 1956).

in rats following parenteral proteases. Gordon and Ablondi[7] have reported subsidence of mustard-oil edema of the rabbit eye following intravenous streptokinase. We[3] have reported reversal of mustard-oil induced edema of the rabbit nictitating membrane following intravenous trypsin (FIGURE 2).

Clinically, subsidence of inflammatory edema consistently follows parenteral protease therapy.[8] This phenomenon is perhaps best demonstrated in patients with cellulitis and acute thrombophlebitis (TABLE 1, FIGURE 3).

(2) *Viscosity and fibrin content of exudates.* Sherry[9] has shown that the local injection of streptodornase into sites of purulent exudation produces a rapid degradation of the desoxyribonucleoprotein and is often associated with a striking clinical improvement of the local area of infection.

Ungar and Ginsberg[10] have described the effect of parenteral trypsin on the tissue response to implanted surgical gut in the liver. In rabbits treated with parenteral trypsin the ring of granulation tissue surrounding the surgical gut was significantly narrower than that of the controls.

My group[3] has studied the effect of parenteral trypsin on croton-oil abscesses in rabbits. Saline-treated rabbits developed thick purulent exudates after 48 to 72 hours. Trypsin-treated rabbits showed marked clearing of interstitial edema and thinned inflammatory infiltrates. In another study in the rabbit we showed that blood clots formed, but that their viscosity dropped 20 centipoises following *in vivo* trypsin. In a third

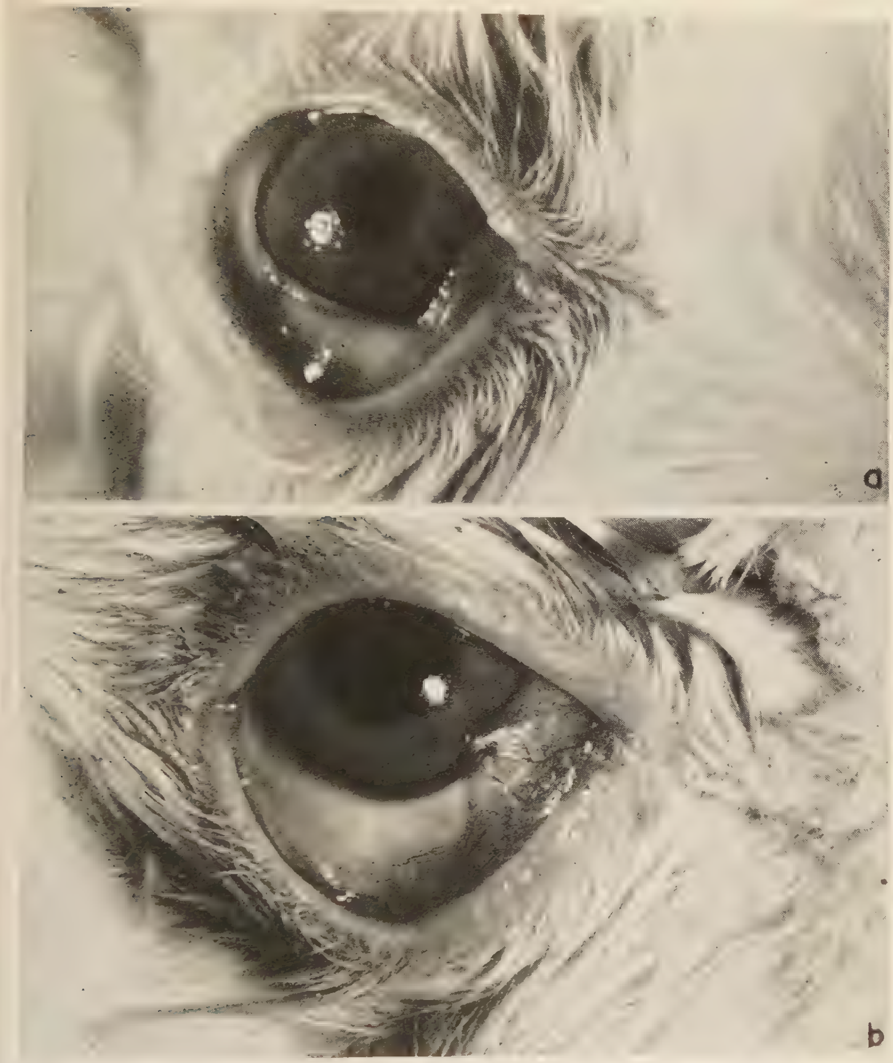

FIGURE 2. Mustard-oil induced acute inflammation of the rabbit eye. (a) Before treatment. (b) Following 5 intravenous infusions of trypsin, 30 mg. each (*Surgery*, vol. 39, p. 426, 1956).

study, empyema and lung abscesses produced in rabbits by injection of carbon tetrachloride and *Staphylococcus aureus* responded to administration of trypsin (FIGURE 4).

Clinically, the exudates of patients with long-standing localized infection show decreases in viscosity and in fibrin content following parenteral protease therapy. This is well exemplified in the response of patients with bronchopleural fistula or leg ulcer (FIGURE 5) to intramuscular injections of trypsin.[11]

TABLE 1
ANTICOAGULANTS COMPARED WITH TRYPSIN IN THROMBOPHLEBITIS

	Superficial thrombophlebitis	Deep thrombophlebitis
Duration of fever		
I.M. trypsin	24 to 36 hours	24 to 36 hours
Anticoagulants	5 to 7 days	3 to 4 days
Incidence of emboli		
I.M. trypsin	0	1.2%
Anticoagulants	2%	13%
Postphlebitic syndrome		
I.M. trypsin	0	about 1%
Anticoagulants	3%	15%
Mortality		
I.M. trypsin	0	1*
Anticoagulants	1	4
Duration of bed rest		
I.M. trypsin	4 to 5 days	4 to 5 days
Anticoagulants	9 to 10 days	10 to 14 days
Total hospitalization		
I.M. trypsin	7 to 8 days	7 to 8 days
Anticoagulants	16 to 20 days	17 to 21 days
Relief of pain		
I.M. trypsin	2 to 10 hours	2 to 24 hours
Anticoagulants	3 to 5 days	3 to 5 days
Loss of time prior to return to work		
I.M. trypsin	9 days	12 days
Anticoagulants	3 to 6 weeks	3 to 6 weeks
Side effects		
I.M. trypsin	Negligible	Negligible†
Anticoagulants	Bleeding in 2%	Bleeding in 2%
Death due to therapy		
I.M. trypsin	0	0
Anticoagulants	1	1‡

* One patient died of pulmonary embolism following gastrectomy for adenocarcinoma with extensive metastases.

† Pain and induration at injection site 5 to 15 per cent. Less than 1 per cent nausea and vomiting.

‡ Two deaths, proved at autopsy: one due to hemorrhagic pericarditis; one due to massive renal hemorrhage.

(3) *Changes in tissue permeability.* Beiler and Martin[12] investigated the possibility that the anti-inflammatory effect of proteolytic enzymes might be due, in part, to their effect on tissue permeability. When isolated connective tissue was used, trypsin had no effect. However, following reduction in the permeability of connective tissue by a mixture of plasma and thrombin, the addition of trypsin tended to restore permeability. These workers also reported that *in vitro* trypsin and other proteolytic enzymes enhanced intracutaneous spreading of dye in rabbits. This finding was related to an increase in tissue permeability. My group[3] reported a 50 to 70 per cent

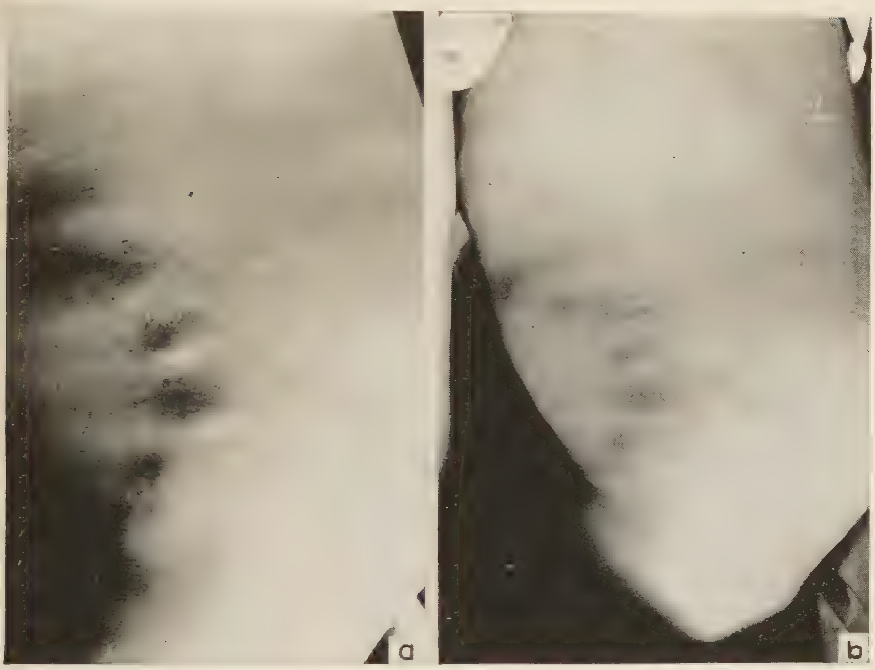

FIGURE 3. Acute thrombophlebitis. (a) On admission. (b) After 48 hours on intramuscular trypsin (*Surgery*, vol. 36, p. 1090, 1954).

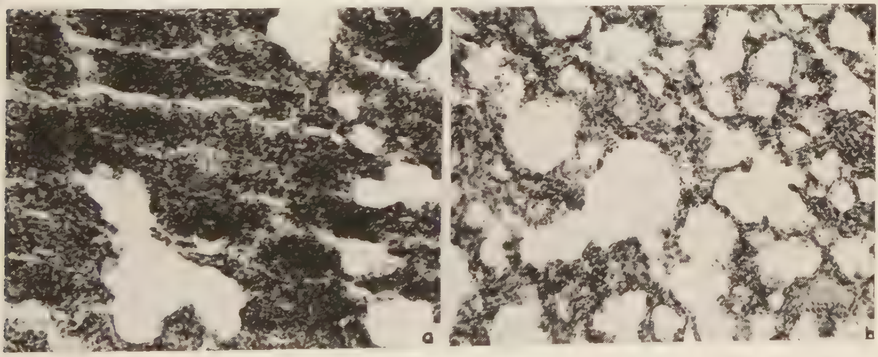

FIGURE 4. (a) Rabbit lung showing diffuse pneumonitis in a control. (b) Trypsin-treated.

increase in area of dye spread in rabbits treated with trypsin, chymotrypsin, or Varidase, as compared with controls. Hechter,[13] who also tested the spreading effect of trypsin in rabbits, found that capillary permeability was increased, as shown by the local accumulation of trypan blue in treated areas following intravenous administration of the dye. The dye spreads in trypsin-treated areas were uniformly greater than in the saline controls and were

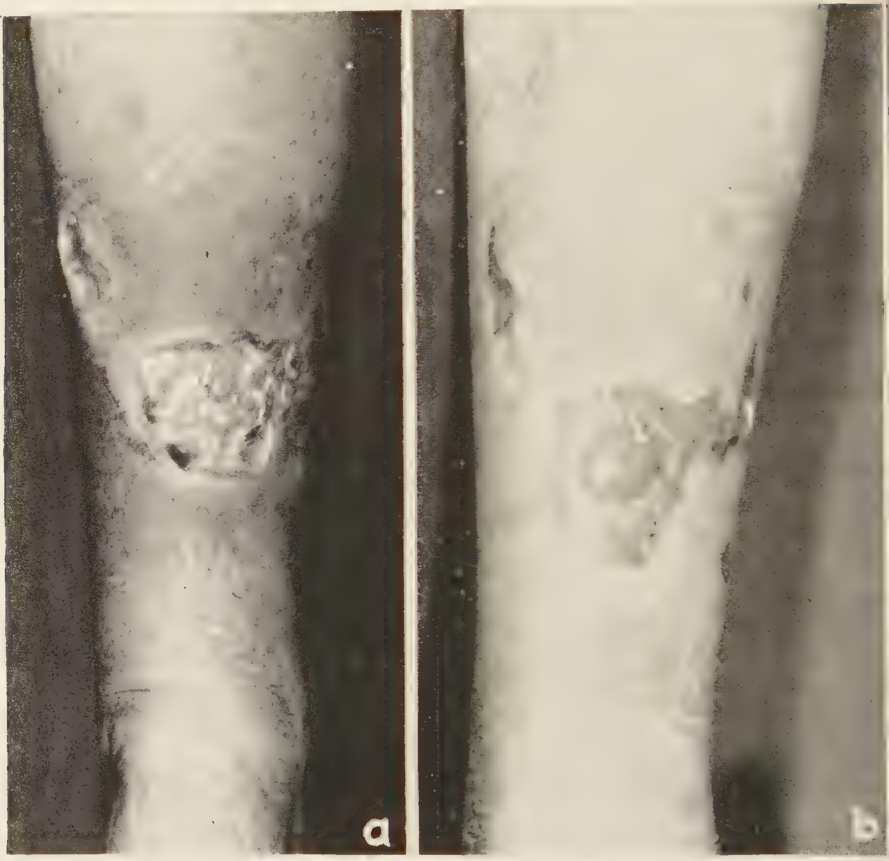

FIGURE 5. Leg ulcer. (a) Before treatment. (b) After 15 days on intramuscular trypsin (*Surgery*, vol. 36, p. 1090, 1954).

"significant." Hechter further observed that trypsin produced in 120 min. an area of spread that appeared to be somewhat greater than that produced by hyaluronidase.

Day[11] has recently reported that perfusion of connective tissue using plasma in which clotting was actually in progress resulted in a marked decrease in permeability. This was apparently due to the formation of partially polymerized fibrin. Day believed that there was subsequent impaction of fibrin macromolecules in interstices of the connective tissue and consequent interference with permeability when clotting was permitted to proceed to completion. When Day added streptokinase to a reaction mixture consisting of partially polymerized proteins undergoing active coagulation, there was a significant increase in permeability. Using trypsin, Beiler and Martin[12] have confirmed this study. These studies suggest a relationship of tissue permeability, proteolytic phenomena, and the size and configuration of protein macromolecules in inflamed tissue.

A basic derangement of homeostasis in inflamed tissue appears to be impairment of the dynamic equilibrium between viscid, highly polymerized protein substrates and the rate of proteolytic hydrolysis. One may now hypothesize that progressive inflammation is characterized by exceedingly long-chained, highly polymerized protein macromolecules, by suppressed proteolysis, and by decreased tissue permeability. Regression of inflammation appears to be characterized by increased tissue permeability, by accelerated proteolysis, by depolymerized protein substrates evidenced by decreased viscosity of protein-containing exudates and by intravascular, intralymphatic, and interstitial fibrinolysis.

The apparent interrelationship between augmented proteolysis, decreased molecular chain length, and increased tissue permeability suggested to me the possibility that trypsin could be administered buccally. Accordingly, a trypsin tablet in a high-molecular weight glycol was designed.[15a, 15b] It is gratifying to report that buccally administered trypsin appears active as an anti-inflammatory antiedemic agent.

Indications for Parenteral Protease Therapy

The basic aims of parenteral protease therapy were recorded in our first paper[8] as follows: (1) to depolymerize long-chained protein molecules; (2) to decrease the viscosity of encapsulated fluid; (3) to augment phagocytosis; (4) to enhance the biochemical process of liquefying purulent debris and inspissated, thick, viscid mucus; and (5) to cause reversal of acute inflammation.

In this early paper we find this statement: "Clinically, the cardinal indication for trypsin is acute inflammation, regardless of etiology." Although this sweeping contention has not been challenged, it is perhaps advisable, in the interests of clinical caution and conservatism, to incorporate antibiotic therapy in addition to enzymes in the treatment of inflammation of bacterial origin.

Contraindications

Hepatic and renal insufficiency and hemorrhagic or shocklike states constitute absolute contraindications to parenteral enzyme therapy.

Although such therapy is not contraindicated, there is no physiological or rational basis for its use in edema of noninflammatory origin, namely, the nephrotic syndrome or congestive heart failure.

Side Effects

The type, frequency, and severity of side effects following parenteral enzyme therapy are related to dosage, route of administration, and duration of treatment. Pain at the site of injection, varying from mild to severe, may be encountered. Whether this is a function of the pain threshold of the patient, the actual injection, the enzyme per se, or the sesame vehicle employed is debatable.

Dosage

The recommended intramuscular dosage of Parenzyme* (trypsin) is 1 cc. (5 mg. trypsin) every 8 to 12 hours for the first week of illness. The recommended dose of Varidase† is 10,000 units streptokinase in 0.5 cc. saline twice daily for from 3 to 6 days. Chymotrypsin (0.5 cc.) is given twice daily for from 3 to 7 days.

Comment

Previous workers have isolated biochemical substances related to the initiation and maintenance of the inflammatory reaction, for example, V. Menkin's "factors," Sir Thomas Lewis's histamine, and the "specific polypeptides" of W. G. Spector and E. S. Duthie. Studies described in this paper permit at least partial biochemical characterization of the reverse phenomenon, subsidence of the inflammatory reaction. These data suggest the importance of augmenting local proteolytic phenomena or, at best, of antagonizing locally suppressed proteolysis, in order to reverse inflammation. In the final analysis, it is restoration of local blood flow in small caliber vessels that constitutes the *sine qua non* for reversing inflammation or, stated differently, for preserving tissue viability despite an acute inflammatory reaction that would otherwise result in suppuration or necrosis.

In a manner yet to be elucidated, parenterally given proteases seem to augment depolymerase activity in inflamed tissue. The definitive experiment demonstrating this phenomenon not only remains to be designed, but constitutes one of the most challenging problems in the field of molecular biology. However, we can at least delineate the level at which further intensive study must be concentrated. V. Menkin has emphasized the importance of intravascular and intralymphatic fibrin deposition in inflammation. Our studies stress the importance of alterations in the geometric design of protein macromolecules, of permeability changes, and of proteolytic inhibition long before the formation of fibrin becomes microscopically apparent in inflamed tissue. Our findings are much more in keeping with those of T. D. Day, W. G. Spector, or R. Dubos, who have emphasized biochemical determinants, including the levels of oxygen, carbon dioxide, *p*H, K, lactic acid, keto acids, the extent and rate of glycolysis, and the molecular chain length of pharmacodynamically active polypeptides. These studies are concerned with the degree and lability of fibrin polymerization, rather than with its morphological distribution.

In the past, emphasis has been placed on the presence of irreversible fibrin thrombi in large-sized vascular and lymphatic vessels, but too little attention has been devoted to apparently reversible physiological and biochemical abnormalities in tissue undergoing inflammatory changes prior to extensive deposition of fibrin and the superimposition of total ischemia. As some of our experiments have demonstrated, despite the persistence of thrombi in major vessels, the ischemia of acutely inflamed tissue can be overcome by activating proteolytic mechanisms for removing extravascular exudative barriers to the flow of blood in smaller compressed, but nonthrombotic, blood vessels. Therein lies the importance of proteolytic enzyme therapy in clinical medicine. It is not the presence of vascular thrombi that concerns the clinical enzymologist dealing with the problem of reversing inflammation; instead, the important consideration is whether, despite the presence of vascular thrombi, tissue viability can be preserved and maintained by decreasing perivascular inflammatory infiltration. Modern parenteral protease therapy, by treating the acutely injured tissue rather than the thrombosed vessels, seems to provide a simple, safe, and effective means for achieving this objective. If parenteral protease therapy is instituted sufficiently early in the course of an evolving inflammatory reaction, it may be possible to reverse such interstitial abnormalities as edema, viscous deposits, tissue impermeability, and ischemia. Indeed, such end results of acute inflammation as tissue suppuration, necrosis, or infarction may be prevented.

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† Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

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Discussion of the Paper

J. M. MILLER (*Veterans Administration Hospital, Fort Howard, Md.*): Dr. Innerfield certainly deserves great credit for first demonstrating that trypsin will reverse inflammation and cause resorption of edema. His work was done with trypsin in sesame oil. We used streptokinase to achieve the same results. We then tried trypsin in saline. Because of rapid deterioration, the solution was made up freshly and used immediately. We gave this trypsin preparation intramuscularly twice a day for three days initially and then in some cases for a longer period. At first we used 2.5 mg. in 1 cc. physiological saline. In 13 of these patients good results were obtained. They were poor in 4. These were individuals on the general surgical service with a wide variety of conditions. The blood supply was poor in 1 of these patients and 2 of them were apparently not given enough trypsin. We therefore increased our dosage to 5 mg., given twice a day, and obtained good results in all in this group. The basic rules are the same no matter what proteolytic enzyme you use. I cannot overemphasize the fact that an antibacterial drug must be given whenever any of these proteases are used intramuscularly. As for the pain and tenderness associated with the use of Varidase, we would see about a 60 per cent incidence of pain and tenderness with Varidase intramuscularly, an extremely low incidence when purified streptokinase was used, and a minimal amount of tenderness and pain when trypsin was used. It may well be that changing the vehicle from sesame oil to saline was responsible for this observation. The areas of inflammation produced by sesame oil and trypsin described by Innerfield were not observed by us. I caution all who use the enzymes to be certain that they are placed intramuscularly. If you put streptokinase or trypsin under the skin, some of your patients will experience a reaction similar to the one Connell has mentioned. We did not see chills, cyanosis, or allergic responses. We did not see any bleeding phenomena or changes in white cell count, nor did we find significant prolongation of prothrombin time. We are ignorant of the mechanism by which trypsin produces the beneficial effects.

MARTIN FISHER (*New York, N. Y.*): We have been using parenteral trypsin for five years, thanks to the stimulus of Dr. Innerfield. We have had experience with some 500 cases of thrombophlebitis, superficial and deep. With the acute superficial thrombophlebitis, particularly the saphenous, we hav been getting results of 70 to 80 per cent improvement that we could attribut to parenteral trypsin. Our problem is the deep ileofemoral phlebitis, i which we feel that we do not alter the course of the disease with parentera trypsin. In some of our recent cases we have even noticed an increase o thrombosis and of the edema following the use of parenteral trypsin. Th question I should like to ask is, how long do you keep these patients o parenteral trypsin before noticing relief of edema? In most of our deep thrombophlebitic cases, treated in the acute phase or many months later, we find the edema is permanent and irreversible.

I. INNERFIELD: We, too, have been unimpressed with the results in deep thrombophlebitis. However, the object of my paper was to stress not the thrombolytic but the anti-inflammatory aspects of enzyme therapy. In this difficult clinical problem of deep thrombophlebitis there are frequently no signs until the patient suddenly has a fast pulse and develops classic signs of pulmonary embolism. The inflammatory component associated with deep thrombophlebitis is not adequate for one to expect a good reaction to trypsin administration. We have recommended, and we continue to recommend, the use of these agents only in cases of thrombophlebitis in which there is a brisk edema and a pronounced inflammatory component. Regarding postphlebitic syndrome and the inhibition of such factors as persistent chronic edema, my own experience has been that there is some hope for the individual, but I doubt very much whether the amount of improvement justifies the prolonged period of injections required.

THE THERAPEUTIC EFFECTIVENESS OF TRYPSIN IN THE TREATMENT OF THROMBOPHLEBITIS

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The role of proteolytic enzymes in clinical medicine has now been clearly established in certain instances and their use is no longer controversial.[1-15] The local application of these enzymes for the enzymatic debridement of necrotic wounds and ulcers has been generally accepted as a proper and effective form of therapy.[10] In addition, despite the incidence of significant side reactions, the instillation of these enzymes into closed spaces has also been advocated in order to promote liquefaction of certain types of exudates and thereby to prevent the formation of organized crippling fibrotic tissue.[10, 15, 17, 18]

The parenteral use of proteolytic enzymes, however, has not been uniformly considered as a procedure of choice in the therapy of disease entities, especially when administration of these enzymes at one site is used to achieve a therapeutic effect elsewhere.[16, 22] In other words, the systemic use of proteolytic enzymes has not found the uniform favor that has been attained by the instillation of these enzymes primarily for their local debridement effects.[23] Many investigators feel that the reported experimental studies on the systemic administration of trypsin do not justify the clinical use of this enzyme parenterally,[16] while others believe that the dose employed could not be of sufficient magnitude to induce any significant therapeutic effect.[19-21] On the other hand, it has been established that the serum of humans contains certain antienzyme substances that detoxify and, in many cases, inactivate parenterally administered proteolytic enzymes. This is particularly true in the case of trypsin, for which a definite measurable titer of antitryptic activity can be determined in human sera.[19] If sufficiently large doses of this enzyme are given to overcome the endogenous antienzyme effect, then such severe side reactions as peptone shock, coagulation defects and, paradoxically, thrombosis may occur.[24] These observations have accounted in part for the opinions expressed by many investigators that trypsin has no place in clinical medicine.[19, 21]

On the other hand, there has accumulated significant and extensive clinical evidence that proteolytic enzymes, such as trypsin, do have a profound beneficial action in a variety of clinical disorders.[4, 25-28, 36] Although most of this evidence is clinical and lacks the objectivity of experimental data, it is difficult to dismiss the glowing reports that have appeared in the current literature.

Thus, previous studies have indicated that trypsin was highly effective in 75 to 80 per cent of all cases of thrombophlebitis and phlebothrombosis.[5, 8, 9, 26, 29] The severity and extent of the thrombophlebitis was rapidly ameliorated and the accompanying disabilities alleviated.[27] Frequently the clinical response was observed after the initial administration of intramuscular trypsin and it often persisted for from seven to ten days.[36] The beneficial clinical response has been attributed to a nonspecific antiphlogistic or a fibrinolysokinetic action rather than to a specific thrombolytic action. Many investigators have theorized that the beneficial effects of trypsin are due not to its proteolytic action, but to the activation of the conversion of profibrinolysin to fibrinolysin.[30, 31-35]

$$\begin{tikzcd}
\text{Fibrinokinase} \arrow[r] \arrow[d] & \text{Antifibrinokinase} & \\
\text{Profibrinolysin} \arrow[r] & \text{Fibrinolysin} \arrow[d] & \text{Antifibrinolysin} \arrow[l] \\
& \text{Fibrin} \arrow[r] & \text{Degradation products}
\end{tikzcd}$$

This enzyme system is usually in constant equilibrium in the homeostatic individual. However, such factors as injury to tissue, burns, hemorrhage, or anxiety may produce an increased level of tissue fibrinolysin, sometimes accompanied by an increased blood level.[32] Simultaneously, the titer of antifibrinolysin rises and excessive hemolytic reactions and bleeding thus are averted. This regulating mechanism of the inactivation of fibrinolysin by antifibrinolysin may well be under the control of the pituitary-adrenal cortical axis (ACTH and cortisone).[31] Untoward increase in antifibrinolysin may be a factor in precipitating thrombophlebitis.

The role of trypsin in the above scheme is still a matter for conjecture. However, parenteral trypsin, injected in small amounts, may reduce either the tissue antifibrinokinase or the antifibrinolysin[33] before the endogenous antitrypsin factors inactivate the exogenously administered trypsin. Consequently, at the site of the local tissue level, there is an increase of the needed fibrinolytic activity.[34, 35] Similarly, antitrypsin may be identical to the antifibrinokinase or antifibrinolysin and, when the total amount is reduced by inactivation of a given dose of trypsin, then the equilibrium shifts in the direction of an increased amount of fibrinolysin at the site of inflammation. This may be a significant factor in reducing the propensity for venous sludging and eventual thrombophlebitis. As the concentrations of the enzyme, proenzyme, and inhibitors at the site of trauma are impossible to measure in the intact human being, a double-blind controlled study was adopted as the most feasible method of determining the therapeutic effect of trypsin in thrombophlebitis. In addition, since thrombophlebitis is a self-limiting disease, any therapeutic application of drugs in this clinical entity required completely objective methods of evaluation before any claim of efficacy could be attributed to the drug.

Subjects

All the patients selected for this study were obtained from the medical, surgical, and obstetrical services of a large county hospital. The patients were selected at random and placed alternately in two groups. Each patient received the same quantity of medication for five days; during that time his clinical status was observed by one of the authors and by the house officer under whose jurisdiction he was at that time. In both the placebo- and drug-treated groups, ancillary therapy, involving antibiotics, anticoagulants, elevation of the extremities, and wet compresses was used at the discretion of the house officers. None of the observers knew which medication was the active principle or which was the placebo. If, after five days of therapy, the patient still exhibited acute symptoms, the other medication was administered. Such a change in medication was necessary in five patients, who thus served as their own controls.

The average age of the patients in both groups was 56 years. There were 15 males and 28 females in the controls and 18 males and 21 females in the treated group.

The degree of the phlebitis was classified, as well as the extent of involvement with respect to the deep or superficial veins. A mild case was considered to be one of minimal pain and redness, the size of the thrombophlebitis being small. Such cases occurred mostly in patients following infusions and cut-downs. The severe cases were those with extensive deep and/or superficial vein involvement and with severe constitutional reactions, such as fever, leukocytosis, pain, redness, and swelling. All cases that had experienced a pulmonary infarction were classified as severe.

Methods

After the degree of phlebitis was established, the patient was arbitrarily placed either in the group receiving the placebo or the group receiving the active drug. Crystalline trypsin (25 mg.) was suspended in a 5-cc. vial of sesame oil. Both the placebo and the active medication were refrigerated. Prior to use, they were warmed to body temperature. Both preparations were injected, as directed by the manufacturer, deep intramuscularly, with a completely dry needle and syringe. The placebo and enzyme were not identified, and they were handled in exactly the same manner. The dose administered was 1 cc. (5 mg.) intramuscularly twice a day for 5 days. A second course of therapy was seldom indicated; the first preparation was inactive in only 5 cases and the patients were then placed on the second preparation. The change was made only in those patients whose general status seemed to have become much worse.

All patients were at bed rest. Continuous wet soaks were used on all cases of superficial phlebitis, and anticoagulants were used in therapeutic doses in all moderate and severe cases as well as in the four cases of mild, deep phlebothrombosis.

A five-day course of therapy was considered adequate. Previous studies have indicated that prolongation of the total treatment or an increase of the dose of the enzyme might increase the severity and frequency of toxic reactions without enhancing the therapeutic effectiveness of the preparation.

Results

Seventy-seven patients are included in this study. There were eighty-two courses of therapy altogether because five patients failed to exhibit a response to the placebo initially and were subsequently treated with the enzyme. These patients served as their own controls, as the disease was definitely progressing while they were on the placebo medication, and was abruptly halted when the active preparation was instituted. A pairing of the patients was attempted so that the degree of phlebitis and the site involved were similar in both groups of patients.

The results were classified as poor, good, or excellent. The patients who displayed an excellent response were those in whom an immediate diminution in the constitutional symptoms of fever, pain, swelling, redness, and tenderness occurred. Such a response often appeared within six to forty-eight hours after institution of therapy. At the end of five days, they were all asymptomatic. However, a painless clot was still palpated in the vein. This did not change for an additional five to ten days. At this point we should emphasize that at no time was lysis of the thrombus observed after administration of the trypsin. All patients classified as an excellent response were ambulated on the tenth to fifteenth day and were then discharged under the care of their private physicians. There were two recurrences in this group and they were treated again with the other medication.

A poor result was one in which there was no change in the affected limb at the end of five days or where there was a progression of symptoms. Any patient who experienced a pulmonary infarct while on therapy, regardless of the local improvement, was classified as a poor result.

A good result was one in which there was improvement at the end of five to seven days, but in which the changes were not as dramatic or as complete as in the excellent-response group. The results are shown in TABLE 1.

In the cases of mild thrombophlebitis, 3 out of 10 did poorly on the placebo and 7 out of 10 had a good or excellent response. In the trypsin-treated cases in this group, 6 out of 6 had excellent to good responses. In the severe

TABLE 1
THE EFFECT OF TRYPSIN AND PLACEBO UPON THROMBOPHLEBITIS

Severity of disease	Classification	Clinical Response						
		Poor		Good		Excellent		Total
		Trypsin	Placebo	Trypsin	Placebo	Trypsin	Placebo	
Mild	Superficial	0	3	1	3	4	1	12
	Deep	0	0	1	1	0	2	4
Moderate	Superficial	1	5	3	2	6	1	18
	Deep	1	7	0	4	7	1	20
Severe	Superficial	1	1	1	1	2	0	6
	Deep	0	11	2	0	9	0	22
	Totals	3	27	8	11	28	5	82

cases of phlebitis, most of which involved the deep veins, 14 out of 15 (93.5 per cent) had a good or excellent response with trypsin; 11 out of 15 had an excellent response (73.5 per cent), while only 1 case had a poor response (6.8 per cent). In the group of severe cases who were placed on the placebo medication, in addition to other accepted measures for therapy of phlebitis, 12 out of 13 patients did poorly (92.5 per cent), while only 1 out of 13 cases experienced good results (7.5 per cent). No patients in this group had an excellent response.

To summarize the results: of all cases on the placebo, only 5 out of 43 (11.6 per cent) had an excellent response, 11 out of 43 (25.6 per cent) had a fair or good response, and 27 out of 43 (62.8 per cent) did poorly.

Of the patients treated with trypsin intramuscularly, 28 out of 39 (72 per cent) had an excellent response, 8 out of 39 (20.3 per cent) had a good or fair response, and only 3 out of 39 (7.7 per cent) did poorly.

These results are statistically significant, and they indicate that trypsin has a direct beneficial action in thrombophlebitis. The objectivity of the method employed gives adequate credence to this statement.

The one case in the moderate deep thrombophlebitis class that responded poorly to trypsin therapy might deserve more detailed description. The patient was a 73-year-old female admitted to the hospital with a hard, tender, painful cord in the calf of the right leg. She also had moderate rheumatoid arthritis and a varicose ulcer on the right leg. She was placed on trypsin and, at the end of two days, a definite diminution of pain and tenderness was noted and, after five days, no cord was felt. The ulcer had become completely clean and granulations were occurring. Nine days after therapy, prior to discharge from the hospital, the patient died suddenly while walking around the ward. No autopsy was obtained, but the clinical impression was that she had experienced a pulmonary infarct. She was thus placed in the poor-response category despite the dramatic relief obtained locally.

Two other patients exhibited a dramatic response of their thrombophlebitis to parenteral trypsin but unfortunately expired from their primary disease two weeks after therapy with trypsin had been discontinued. In one, carcinoma of the prostate was present, and the other had rheumatic heart disease and auricular fibrillation, with a cerebral embolism causing the sudden demise.

Side Reactions

Only two cases in the treated group complained of severe pain at the site of injection but therapy was nevertheless continued. One patient on the control medication developed severe pain at the site of the injection and generalized hives. He was switched over to the active drug and did well. This reaction was obviously a sensitivity to penicillin, which he had received concomitantly. The vast majority of patients, however, tolerated the medication well without side reactions.

Conclusion

A double-blind controlled study is the most effective method of evaluating the response to any medication in a self-limited disorder. In this study the results indicate that trypsin was highly effective in 72 per cent of the patients, in contrast to 11 per cent for the placebo. A poor response was observed in only 8 per cent of the patients on the active enzyme as compared to 63 per cent on the placebo. This dichotomy of effect between trypsin and the placebo is highly significant and substantiates previous clinical observations on the effectiveness of parenterally administered trypsin in the treatment of thrombophlebitis.

Summary

Trypsin was administered parenterally, in a double blind study, to 77 patients with thrombophlebitis. The results clearly indicate the value of trypsin as a potent therapeutic agent in the treatment of this disease. It is felt that trypsin has an important role in the armamentarium of the physician required to treat thrombophlebitis.

Acknowledgment

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Discussion of the Paper

Clinton H. Thienes (*Pasadena, Calif.*): I wish to congratulate this group of workers on their attack on the problem of enzyme therapy. Until now I have not been convinced that all of these changes that have been reported were more than accidental. However, a blind placebo test like this is very convincing. I think that all of the theoretical objections to this type of therapy have very little basis until the opponents come up with a clinical investigation of this caliber.

THE ANTI-INFLAMMATORY EFFECT OF INTRAMUSCULAR STREPTOKINASE

By Joseph M. Miller

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A more detailed understanding of the mechanics of inflammation has permitted the evolution of a more rational method of treatment. After the studies of Metchnikoff were published,[1] it was believed that phagocytosis was the most important part of the inflammatory reaction. The ingestion of bacteria by phagocytes does not, however, necessarily imply protection for the host. A new approach to the treatment of inflammation during recent years has utilized the forces exerted by the humoral defense and the immunological response of the host, by the antibacterial drugs, and by certain proteolytic enzymes.

The cellulitis seen initially in inflammation may be partly resolved by the humoral defense of the body. Phagocytosis is best effected on a surface where leukocytes can trap bacteria against an opposing surface such as that presented by the wall of the tissue involved or by other leukocytes.[2-4] Surface phagocytosis is relatively inefficient in open cavities such as those found by the peritoneum, the pleura, the pericardium, and the meninges[4] or in the center of an established abscess.[4] Leukocytes are not effective phagocytes in fluid.[4] When an adequate supply of oxygen is lacking, the leukocytes become nonmotile and lose their power of phagocytosis.[5]

The inflammatory reaction may be modified by the immunological response of the host. Two groups of antibodies, classified according to the nature of the antigen stimulating the formation of the antibody, are of concern in the general field of infectious diseases: the antitoxins and the antibacterial antibodies.[6]

The introduction of the antibacterial drugs has permitted tremendous advances in the treatment of inflammation, particularly of cellulitis. The results in the treatment of abscess have not been as spectacular. The action of many of the antibacterial drugs on selected bacteria is greater *in vitro* than *in vivo*. It may be concluded, therefore, that certain factors limiting the action of these agents exist at the site of inflammation. The therapeutic effect of an antibacterial drug depends upon: (1) the type and the number of bacteria present; (2) the susceptibility of the microorganisms; (3) the concentration of the drug at the affected site; (4) the chemical environment at the involved area; and (5) the degree of immunity of the host.

The chemical environment in which the drug acts and the concentration of the drug at the affected site may be changed by streptokinase. To understand how these factors may be influenced necessitates a review of the present knowledge of inflammation.

The acute inflammatory reaction is characterized by excessive local dilatation of the capillaries due to biochemical changes, the nature of which is not definitely known. The capillary endothelium is injured. Permeability is thereby increased, allowing fluid to pass more freely into the tissues. Polymorphonuclear neutrophilic leukocytes and, later, mononuclear cells enter the area of inflammation. In the fluid, fibrin is formed from fibrinogen of the blood stream by the action of thrombin liberated from disintegrated blood platelets. Hughes[7, 8] has demonstrated that the wall of fibrin formed in and about the site of inflammation is remarkably selective in permeability. When filtered under pressure, albumin will pass through, but globulin, containing antibodies, will not. Some bacterial toxins will diffuse slowly, although most products of such origin will not. Bacteria are restrained, but polymorphonuclear neutrophilic leukocytes pass readily. Apparently fibrin usually prevents the spread of bacteria, but protects them from immune bodies in the globulin fraction of the blood serum. The deposition of fibrin in the tissue fluids and the formation of thrombi in the arteries, capillaries, veins, and lymphatics of the area help to localize the infection, but the impairment of circulation of the blood and the inhibition of drainage of fluid from the inflamed area lead to necrosis of the tissue and to the formation of an abscess. White blood cells, trapped in the area, die and deposit desoxyribonucleoprotein. Fibrin and desoxyribonucleoprotein coagulate about the bacteria.

Fibrinolysis, indirectly effected by streptokinase, provides an avenue of attack on one of the components of the inflammatory barrier.[9] Streptokinase activates the plasminogen in the euglobulin fraction of the blood to plasmin. Plasmin causes lysis of fibrin.

Observations in patients with infections and edema indicate that profound effects in reversing these states can be produced by the intramuscular administration of streptokinase. The mechanism of the action is at present unknown and has not been proved by microscopic examinations or chemical analyses. It is assumed that fibrin in edematous fluid is digested, and that the viscosity of such fluid is reduced. The fibrin clots in the local vascular and lymphatic circulations are dissolved, and the edematous fluid is resorbed. The net result is an improvement in the local vascular and lymphatic systems. The humoral defenses, the immunological forces, and the antibacterial drugs can reach the bacteria in larger concentrations and therefore can combat them more effectively. If the inflammation can be prevented from progressing beyond the stage of cellulitis, all of these defenses can act more effectively, since the products of digestion of bacteria and tissues may provide material for the enhancement of the inflammatory state.

Initially, Varidase*, which contains streptokinase and streptodornase, was used, since pure streptokinase had not yet been manufactured. The effects achieved by Varidase could be ascribed to the action of streptokinase because the observed therapeutic effects were not produced by the intramuscular injection of a pure preparation of streptodornase.

Varidase was dissolved in physiological saline so that the final concentration was 10,000 units of streptokinase per cubic centimeter. The solution must be made fresh daily and should be stored in a refrigerator when not in use.

* Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

A dose of 5000 units of streptokinase in 0.5 cc. of physiological saline was injected intramuscularly twice a day for a minimum of 3 days.[1] Treatment may be given for a longer period if necessary. An antibacterial drug must be given concurrently. Beneficial effects on the inflammatory reaction and edema were seen by the end of the second day. An aggravation of the infection was not seen in any of the patients. About 60 per cent of the patients complained of a moderate degree of pain at the site of injection. The pain disappeared in from 24 to 48 hours after treatment was discontinued. It is believed that the pain and tenderness might have been due to a reaction to foreign proteins. In patients with granulating wounds, healing was hastened by the reduction of the surrounding edema. Delayed healing was not observed. Ten per cent of the patients showed a rise in temperature attributable to the streptokinase. No significant changes were seen in the white blood cell count, the differential count, or the prothrombin time. Fibrinolysis, as determined by the whole-blood technique, was not observed in periods up to 72 hours after the fifth injection of streptokinase on the third day of treatment. No hemorrhage or formation of hematomas or petechiae was seen; granulomas were not seen in any of the patients; chills, cyanosis, or an allergic response were likewise absent. The administration of an antihistaminic drug was not necessary. Streptokinase should not be given to patients known to have defects in the clotting mechanism. In 64 patients with various types of infection and edema, the results were excellent in 45, good in 15, and poor in 4. In the 4 poor results, the blood supply was deficient in 3 patients and insufficient treatment was given to the fourth.

Five patients with acute thrombophlebitis lost the pain, tenderness, and overlying redness and edema in about 48 hours. The palpable cord in the vein lasted for about 7 days. In 6 comparable patients treated in the same manner, but without streptokinase, the pain, tenderness, and edema required about 15 days to disappear.

Patients subjected to craniectomy for brain tumor were treated with the intramuscular injection of streptokinase. If the operation was emergent, treatment was started just before the procedure, but if the operation was elective, the patients were treated for 2 days in advance. No increase in the amount of hemorrhage at the time of the procedure was seen in these patients. The clinical course of the patients was most encouraging, suggesting that the cerebral edema normally consequent on operative trauma was less than usually seen.

In 5 patients with acute epididymitis, the pain and tenderness disappeared within about 48 hours.[10] The area of inflammation became softer in about the same period, with complete resolution at about 5 days. In 6 patients with acute epididymitis treated similarly, but without streptokinase, the pain and tenderness gradually subsided over a period of five to seven days. A much more gradual resolution of the inflammation occurred, with the complete stage being reached in about 9 days.

A patient with mumps and a complicating epididymo-orchitis obtained an excellent result from the intramuscular injection of streptokinase.[11] The administration of tetracycline hydrochloride did not produce any effect.

The intramuscular administration of streptodornase had no beneficial effect. The intramuscular injection of streptokinase produced rapid improvement in symptoms and signs.

A patient with a severe right frontal sinusitis showed an excellent resolution of the inflammatory reaction following the intramuscular injection of streptokinase.[12] The symptoms and signs of the inflammation disappeared rapidly.

Of 5 patients who had enucleation of the eye and who were given streptokinase intramuscularly, 1, who had received the drug after operation, was fitted with a conformer on the twenty-third postoperative day, but the 4 patients who had been given streptokinase before the enucleation was done were fitted with a conformer 4 to 7 days postoperatively.[13] In the patient whose healing was delayed, the lids were sutured after enucleation because edema of the conjunctiva was severe. The lid sutures were subsequently removed because of severe pain and prolapse of the conjunctiva between the sutures. Streptokinase given intramuscularly produced a remarkable decrease in the edema, but the prolapse of the edematous conjunctiva became relatively more pronounced. A Snellen suture was placed in the lids, but the suture came out spontaneously. A new Snellen suture was placed and was subsequently removed. A considerable improvement in results was noted when the patients treated with streptokinase before the enucleation was done were compared to patients not so treated. In 16 patients not treated with streptokinase who had enucleation with insertion of plastic-sphere implants, all had moderate to severe edema of the conjunctiva. Five patients had a prolapse of the conjunctiva; of these, 2 required secondary lid closure with Snellen sutures. In all patients, fitting of a custom-made conformer was delayed until at least the fourteenth postoperative day. The fitting of a prosthesis at as early a date as possible is important because it helps to reduce the number of mild contractual deformities of the superior fornix and of the inferior fornix ot the conjunctiva and to prevent untoward psychological changes.

Two patients who had inflammation and edema following the extraction of teeth received benefit from the intramuscular injection of streptokinase.[14] One of these patients illustrated the excellent effect obtained by using streptokinase before the extractions.

Since about 60 per cent of the patients who received streptokinase intramuscularly complained of pain and tenderness at the site of injection, some method of preventing this reaction was sought. The use of solutions of procaine hydrochloride as a solvent for the streptokinase was attempted. In 6 patients with various types of inflammation and edema treated with 5000 units of streptokinase dissolved in 1 cc. of a 1 per cent solution of procaine hydrochloride, pain and tenderness were not alleviated, although the results of treatment with streptokinase were satisfactory otherwise. Similar results were obtained in 6 patients with various types of inflammation and edema treated with 5000 units of streptokinase dissolved in 1 cc. of a 2 per cent solution of procaine hydrochloride.

The pain and tenderness associated with the intramuscular injection of streptokinase as Varidase is a major disadvantage to the use of the drug in the treatment of inflammation. The availability of streptokinase purer than the original drug has permitted further advance in the therapeutic management of such patients. Eighteen patients have now been treated with the purified preparation, with excellent therapeutic results. Only one patient had slight tenderness and pain associated with the intramuscular injection of streptokinase.

Elevation of the temperature, an effect that had been seen in about 10 per cent of the patients given Varidase intramuscularly, was found in one patient when the purified preparation was used. The temperatures observed in the other patients were due to the disease present and were not increased when the purified streptokinase was given intramuscularly. One patient in the group given the purified streptokinase had an allergic reaction to the drug. The patient was a 38-year-old white man who had an infection of the first finger of the right hand. He had a history of rheumatic fever and was currently suffering from rheumatoid arthritis. Five thousand units of purified streptokinase was given intramuscularly. No pain, tenderness, or granuloma was noted at the site of injection. The patient complained of chills and malaise. His temperature rose to approximately 103° F. in about 11 hours and returned to normal about 24 hours after the maximal temperature was reached. About 6½ hours after the administration of the purified streptokinase, the patient had a severe pain in the affected joints. No further injections of the drug were given at this time. Subsequently, a provocative of the same drug was given intramuscularly and a reaction similar in all respects was obtained. Later 100 units of purified streptokinase in 0.1 cc. of physiological saline was injected intradermally into the volar surface of the right forearm and a control of 0.1 cc. of physiological saline in the volar surface of the left forearm. A marked reaction was obtained from the injection of the purified streptokinase while the control injection gave a normal reaction.

Comment

The observations concerning the intramuscular use of streptokinase as Varidase and the data more recently obtained from the use of purified streptokinase are summarized as follows: (1) most patients showed a beneficial effect after 24 hours; (2) no aggravation of infection was seen; (3) no delay in healing of wounds was noted; (4) 10 per cent of the patients given streptokinase as Varidase had a rise in temperature of 2° F. to 3° F. that was easily controlled by medication, but such elevations in temperature were observed in only one patient when the purified streptokinase was used; (5) no changes in the peripheral blood picture were found; (6) no significant alteration of the prothrombin time was detected; (7) no fibrinolysis was seen; (8) some pain and tenderness were noted at the site of injection in about 60 per cent of the patients given streptokinase as Varidase, but only one patient in 18 had a mild degree of pain and a slight degree of tenderness when the purified streptokinase was used; (9) no hemorrhages, hematomas, or petechiae were found; (10) no granulomas were detected at the site of the injection; (11) no chills, cyanosis, or allergic reactions were seen in the group given streptokinase as Varidase, but one allergic reaction was seen when the purified streptokinase was used in a patient with a history of a collagen disease and during an attack of a collagen disease.

The usual course of treatment with streptokinase is 5000 units in 0.5 cc. of physiological saline administered intramuscularly twice daily for a minimum of 6 doses. Treatment may be continued longer if necessary. Streptokinase may be given preoperatively where considerable edema is expected postoperatively. An antibacterial drug must be administered when streptokinase is given intramuscularly. Streptokinase should not be given to patients known to have defects in the mechanism of clotting. Streptokinase should not be administered to patients with a history of one of the collagen diseases or during an attack of one of these diseases.

Summary

Streptokinase given intramuscularly produces a modification of the inflammatory reaction. Treatment with streptokinase given intramuscularly must be accompanied by the administration of an antibacterial drug. The use of streptokinase given intramuscularly is not a substitute for sound surgical treatment. Further investigation into the use of streptokinase given intramuscularly is indicated.

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Discussion of the Paper

G. Ungar (*U. S. Vitamin Corporation, New York, N. Y.*): I have yet to learn the answer to one question: What evidence is there to show that this is a specific action of these enzymes? Some of us are old enough to remember that about twenty-five years ago nonspecific protein therapy appeared to give results as good as those obtained with trypsin and streptokinase. At present there are some pyrogens, for example, one isolated by Westphal in Germany, that have shown excellent anti-inflammatory results. These substances are nonspecific; we do not know the mechanism of their action. It may be enzymatic, but it involves a much more complex situation than the direct effect of a protease that is already diluted when it arrives at the site of action. I should like to ask if anyone has injected any type of foreign protein and observed the results on the types of inflammation that have been reviewed here.

J. M. Miller: Regarding foreign protein, as a part of our preliminary observations we gave a group of patients nothing but purified streptodornase to enable us to reproduce the foreign-body effect. One patient was treated first with streptodornase, without effect, and then treated with streptokinase.

I too am old enough to remember treating people who had inflammation of the eye with boiled milk. My impression is that we achieved results in some of these patients, but that they were not as good as the results that we are obtaining now.

Question: You warned us about the administration of proteolytic enzymes to patients with the so-called collagen diseases. Do you have any other experience with such cases besides the one to which you alluded?

J. M. Miller: This is the only individual who had such severe manifestations, but the results were very dramatic. I believe that the administration of these streptococcal products in patients who have a proved history of rheumatic fever is strongly contraindicated because of the hazard of reproducing changes.

CLINICAL EXPERIENCES WITH CHYMOTRYPSIN

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Since there is little need to review the older literature dealing with the clinical uses of proteolytic enzymes, only recent experiments will be cited. Hendley *et al.*[1] have compared chymotrypsin with trypsin because chymotrypsin shares the fibrinolytic activity of trypsin but has no clotting action on the blood *in vitro* or *in vivo*. Their results indicate that the LD_{50} for chymotrypsin by rapid intravenous injection ranges from 24,000 units kg. in the rabbit to 85,000 kg. in the mouse. These authors have also found that trypsin is considerably more toxic by rapid injection than by slow injection, whereas chymotrypsin shows little difference in this respect, and is less toxic by rapid administration than is trypsin.

Miechowski and Ercoli[2] have demonstrated that chymotrypsin possesses a greater anti-inflammatory activity than does trypsin. These authors used chemical inflammation, anaphylactoid inflammation, ultraviolet radiation, and thermal burns to test the anti-inflammatory activity of both enzymes.

Using the level of blood esterase as an indicator, Hill and Bastian,[3] suggested that the anti-inflammatory effect of parenterally administered trypsin and chymotrypsin is not mediated by the systemic absorption of the enzymes

From these experimental data it appears that chymotrypsin, when administered parenterally, has a wide range of safety, possesses an anti-inflammatory action, and does not appear to be absorbed into the circulation in any measurable quantity. The fact that the enzyme is not absorbed in any measurable amount has made the clinical investigation somewhat difficult, for there is then no acceptable physiological basis for the anti-inflammatory activity. In spite of this, we initiated a clinical study, which is being continued, and the results of which are reported in this paper.

Jenkins[4] has already reported his results from the use of chymotrypsin[*] parenterally in various ophthalmic diseases such as conjunctivitis, uveitis, hyphemia, and retinitis. He also has used chymotrypsin to hasten the absorption of hematomas of the periorbital tissues. Since there are no definite methods for the measurement of chymotrypsin, the results obtained from its use can be judged only by clinical experience and by indirect comparison with other therapeutic regimens. By these standards, chymotrypsin definitely shortens the course of ophthalmic inflammations and hastens the absorption of blood in the tissues. The acceleration of absorption of blood seen in hyphemia has led several ophthalmologists to use chymotrypsin both preoperatively and postoperatively in cataract surgery, and they have reported excellent results.

The value of the parenteral use of chymotrypsin to hasten the absorption of blood in the tissues is dramatically shown by the following case. Th patient, a 50-year-old farmer, had his head caught in a hay baler and was first seen 12 hours after the accident. He had sustained a basal skull fracture and a fracture of the nose, and a hematoma extended from below the eyes up to the vertex. The eyelids were edematous and approximately 1/4 in. thick. Chymotrypsin was injected, 1/2 cc. 3 times a day; at the end of 40 hours practically all of the edema had disappeared and most of the hemorrhage had been absorbed. The patient had regained consciousness, and one eye was open and the eyelids of the other eye could be separated with ease. This patient has had a fairly uneventful recovery except for a disturbance in equilibrium. Again, there is no means of ascertaining whether chymotrypsin had an effect on the injury to the brain, but it is certain that the absorption of the edema and hemorrhage hastened the recovery.

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[*] The chymotrypsin suspension used in these studies is Chymar, The Armour Labora tories, Kankakee, Ill. Each cubic centimeter contains 5000 units of proteolytic activity

We have recently received a report of treatment with chymotrypsin in a series of cases of chronic asthma complicated with sinusitis; 5 of the 6 patients have shown remarkable recovery, and 2 of these histories will be given in detail.

H. S., a 45-year-old female, had been under treatment since 1952 for perennial allergic rhinitis, hay fever, and a seasonal asthma. Her chief complaint was nasal clogging, of which she complained bitterly at every visit. She was treated for one year with injections of extract of rag weed, dust, orris, and fungi, with very little improvement, and the treatment was discontinued.

The patient returned in February 1956. In the interval she had had continued nasal clogging, with a thick discharge, soreness in the nose, and frequent nasal infections. She reacted to and was treated with extracts of the various antigens, and in addition she was given 1/2 cc. of chymotrypsin in oil. One week later she returned and reported marked improvement. Chymotrypsin was administered again. Two days later she reported such a marked improvement that treatment with chymotrypsin in oil was continued.

Although she had previously reported that the nasal symptoms had been present during the entire year, after the last injection of chymotrypsin she was breathing freely and there was no nasal obstruction. The injections of chymotrypsin were subsequently discontinued and she progressed fairly well. This patient served as her own control because there were two separate treatment periods, the first highly unsuccessful and the second, during which she was treated with chymotrypsin, very successful.

S. Y., a 13-year-old female, had been under treatment since December of 1955 for seasonal hay fever and perennial rhinitis. In September of 1956 she developed an upper respiratory infection with a purulent nasal discharge and a severe nocturnal cough. Various medications were tried, without result. She was injected with 1/4 cc. of chymotrypsin in oil, and two days later she reported that the cough had stopped. She was then injected with 3/4 cc. of chymotrypsin. She again reported steady improvement and it was decided to use 1 cc. of the suspension. Three more injections were given and since then she has been almost completely well.

We have received another report of a series of 50 patients with chronic asthma who were treated as ambulatory patients. These patients were given

½ to 1 cc. of the suspension of chymotrypsin in oil every day or every other day for 10 to 14 days. Practically all of these patients have reported that the asthma has gradually disappeared, that their respiratory efforts are less forceful, and that they can carry on in their usual occupations with comparative ease.

One of the greatest fields of usefulness of parenteral enzymes is in traumatic injuries. Recently I have had the opportunity to discuss the subject with three of the trainers of college football teams. Because of the pressure of modern football and the insistence of the coach on having players available, trainers are always interested in hastening the reduction of edema and hematomas. One of the teams I visited gave a most interesting demonstration of the effectiveness of chymotrypsin in hastening the absorption of hematomas. Last year their star end was incapacitated most of the season because of a large bruise over the right iliac crest. This bruise had caused considerable spasm of the gluteal and abdominal muscles and had prevented him from running and therefore from playing. This year early in the season he again had a similar injury that was promptly treated with chymotrypsin. The player told me that thirty-six hours after the injection of chymotrypsin he had no muscle spasm and was perfectly willing to return to practice. I had the opportunity to watch this team in action and on the first play of the game he caught a forward pass and ran fifty-five yards for a touchdown.

At another large university one of the team's most necessary players received a very similar injury. He, too, reported that most of the bruise had disappeared and that, 3 days after treatment, he felt well enough to engage in scrimmage. He played a good game on the following Saturday.

Another troublesome injury in football players is what is commonly called a Charley horse—a large hematoma within a muscle mass. Such an injury will subside on treatment with heat, whirlpool baths, and ultrasonic therapy. However, many of these hematomas absorb so slowly that calcium is deposited and the elasticity of the muscle is damaged. The early parenteral administration of the enzymes hastens the absorption of the hematoma, relieves the spasm of the muscle, and apparently prevents the formation of fibrous tissue and calcium deposits.

Trainers of football players are also using Chymar to accelerate the reduction of edema following strains and sprains of joints and, while they perhaps do not entirely believe in immobilization of the joint, they do apply layers of adhesive tape to support the affected joint. Most of them believe that a rapid reduction of edema will permit early healing of the injured tendons; therefore they feel that the enzyme does hasten healing.

The team physician for a high school reports that the team's leading halfback received a severe muscle injury that was treated with the suspension of chymotrypsin in oil. In the physician's own words, "This player would have been incapacitated for two months, but was returned to active playing within two weeks."

We have reports of three studies on the use of suspensions of chymotrypsin for the relief of edema in episiotomy wounds. Each patient was given ½ cc of the suspension immediately before leaving the delivery table and a second injection of $\frac{1}{2}$ cc. at the end of the first day postpartum. The investigators have now used this regimen in approximately 250 cases. More than 80 per cent of the patients have reported complete relief from the edema in the wound, and in no case has it been necessary to release the sutures. There have been no reports of delayed wound healing or of need for secondary closure.

We must admit that there have been some failures with chymotrypsin in oil, but there have been remarkably few reports of sensitivity reactions, as manifested by urticaria. However, it is well known that sesame oil is an allergen to some individuals and some allergic reactions are to be expected. We have also received some reports that the injection of the suspension produces some pain; strangely enough, most of these reports have come from athletes. Apparently they are psychologically adjusted to severe injury due to contact sports, but the idea of an intramuscular injection is not well accepted despite the knowledge that healing of their injuries will be facilitated.

In conclusion, from the reports we have had from investigators and from unsolicited reports it may be said that the use of chymotrypsin parenterally hastens the absorption of edema and of blood in the tissues, and that this enzyme acts as a remarkable anti-inflammatory agent. Despite the lack of knowledge as to how or why the parenterally administered enzymes produce these effects, it is evident that parenteral enzyme therapy is a proved addition to our therapeutic armamentarium.

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CLINICAL EXPERIENCE WITH TRYPSIN

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Crystalline trypsin in sesame oil injected intramuscularly has been advocated as a therapeutic agent in a wide variety of conditions.[1] While it was used initially because of its local proteolytic action on necrotic tissue and exudates, later applications involved its antiedema, anti-inflammatory action when given intramuscularly. In bacterial and chemical inflammation, in trauma, and in hemorrhage it hastens the reabsorption of the exudative or extravasated elements into the blood and lymphatic vascular systems. By reducing excessive edema it hastens resolution and healing. The mechanism by which trypsin exerts this action when given intramuscularly is still a subject of discussion.[2, 3] Because of its action against both inflammatory and traumatic edema, trypsin theoretically should be useful in many different conditions. Its effectiveness in practice has been the subject of many investigations. The earliest applications involved its use in the treatment of thrombophlebitis and in limiting the swelling subsequent to rhinoplasty.

In this study the effectiveness of intramuscularly injected crystalline trypsin in the treatment of traumatic injuries was judged. An attempt was made to evaluate its action in the limitation of the reaction to injury as compared to the reaction of controls. The conditions involved were: black eyes, bruises, hematomas, sprains, fractures, and thrombophlebitis. Because the development of edema is so critical when an anastomosis is performed, the prophylactic administration of trypsin was tried to determine if it were clinically effective in hastening healing where an anastomosis had been performed in the common duct or intestine.

Method

Crystalline trypsin in sesame oil was injected intramuscularly deep into the buttock. It was essential that a dry syringe and needle be used and that the needle be long and of large lumen. When these precautions were not followed, the site of injection would become red and painful. Furthermore, extra movement of the needle must be avoided because traumatic injections become painful when trypsin is injected. Five mg. in oil were given every eight hours except in the case of minor injuries, such as black eye, in which the patient was seen only once a day. Most of the patients were hospitalized and routine observations were made. Antibiotics were not administered except when inflammation was present. In the intestinal anastomoses the same routine of intraluminal and parenteral antibiotics was used in both the experiments and the controls.

Results

Black eyes, bruises, and hematomas. Trypsin was administered to study the resolution of the extravasation of blood in injuries sustained by athletes in contact sports or by individuals who had recently been injured. Ordinarily it takes about 10 days for the swelling and pigment to resolve. After treatment with trypsin in 350 cases presenting such injuries, results were good in 318 cases and were questionable in 32 cases (TABLE 1). In the "good" results, swelling and pain disappeared in 24 hours and the hemorrhage resolved in 72 hours. Furthermore, the color changes of the ecchymosis in the skin were different: there was rapid appearance (within 24 hours) of a pink-red color in the skin rather than the gun-metal blue-black seen in the untreated ecchymosis.

Vestiges of subconjunctival hemorrhage remained for as long as 4 to 5 days. The swelling around soft-part injuries disappeared more rapidly than in the untreated cases. The marked subcutaneous edema and hemorrhage seen after rhinoplasty, thyroidectomy, and neck dissections showed remarkable resolution in 24 hours. In 32 of the 350 cases there was poor or no response to trypsin therapy.

Three patients who had intraocular hemorrhage due to trauma showed resolution of the hemorrhage in 3 to 4 days.

Hemorrhage around sprains and fractures. Forty-two patients with sprained ankles were treated with intramuscular crystalline trypsin. Of these, 39 showed a satisfactory response. There is a great advantage in keeping the swelling down at the site of a sprain. It permits palpation of bony landmarks and consequently there is greater ease in following the progress of healing in the joint. Since the pain in these sprains is due to hemorrhage into the ligaments and synovia at the site of attachment to bone and muscle, trypsin therapy causes a rapid reduction of pain as compared with the untreated controls. Seven cases in which there was hemorrhage into and around the knee joint showed that trypsin produced reduction of the swelling and pain in 24 hours. Similar response was obtained in hemorrhage around fracture sites. This rapid reduction in swelling permitted more accurate immobilization of the bones in these fractures and possibly better approximation of the fragments.

TABLE 1

RESULTS OF THERAPY WITH CRYSTALLINE TRYPSIN

Clinical condition	Number of cases	Good results	Poor results
Black eyes, bruises, hematomas	350	318	32
Traumatic intraocular hemorrhage	3	3	0
Sprained ankles	42	39	3
Hemarthrosis knee joint	7	7	0
Fractures with marked swelling	15	15	0
Thrombophlebitis	28	24	4
Intestinal anastomoses	59	54	5
Gastric retention due to malfunctioning stoma	3	3	0
Intestinal obstruction	10	10	0
Ascites due to cirrhosis or carcinoma	53	38	15

In 28 patients in whom thrombophlebitis was encountered, 24 cases demonstrated a 24-hour response to the trypsin therapy.

Intestinal and other anastomoses. During the performance of end-to-end anastomoses a diaphragm is turned in by the rows of sutures placed. The trauma from manipulation, the passages of the needle, and small hematomas due to perforation of small vessels invariably produce edema.. Very often this edema delays the time within which the intestine or duct will permit passage of the contents. This situation was considered an excellent test of the effectiveness of trypsin in reducing edema. In the case of intestinal anastomoses the time of effective peristalsis and passage of gas is easily observed. In 59 cases intramuscular trypsin was administered immediately after operation and treatment was continued for 3 days (TABLE 2). Effective peristalsis occurred about 24 hours earlier than in similar controls, and less distension occurred in 54 cases. This success encouraged a trial in the treatment of intestinal obstruction due to carcinoma and diverticulitis of the colon. In 10 cases of intestinal obstruction, trypsin therapy seemed to be of distinct value. However, in such cases the difficulty of comparison with controls is very great, and this application is mentioned merely as suggestion for further trial in reduction of the inflammatory edema existing around lesions of the large intestine.

Ascites due to carcinomatosis or cirrhosis of the liver. An attempt was made to determine whether trypsin injected into the peritoneal cavity would delay the recurrence of ascites after removal of the ascitic fluid in these two conditions. After removal of the ascitic fluid by paracentesis or laparotomy, 5 mg. of trypsin in saline was injected into the peritoneal cavity. The time of reaccumulation of the ascitic fluid was measured. This was attempted in 53 cases. In 38 patients this therapy slowed the recurrence of the ascites. This effect could be repeated 2 or 3 times, but thereafter the intraperitoneal trypsin was ineffective.

Table 2

Types of Anastomoses in 59 Cases Treated Intramuscularly with Crystalline Trypsin in Sesame Oil

Gastric resection:	
Bilroth I	6
Bilroth II	16
Gastroenterostomy	1
Small intestine end-to-end (adult)	10
Small intestine end-to-end (newborn)	2
Ileotransverse colostomy	4
Colon:	
End-to-end colocolostomy	4
Closure colostomy	2
Anterior resection colorectostomy	14
Total	59

Side Effects of Trypsin Therapy

Extensive discussion with others using crystalline trypsin in sesame oil intramuscularly has revealed certain difficulties in its use. There have been comments on pain, redness, and swelling at the site of injection. This has not been a problem in our study. The pain can be avoided by the careful injection of the substance deep in the muscles of the buttock using a dry, large-bore, long needle. No allergic or febrile responses were noted in this series. Some observers have commented on allergic responses to sesame oil used in the injection. While many investigators have given antibiotics at the same time as trypsin, in this study trypsin has been found to be effective without such addition, and antibiotics were administered only where definite infection indicated such use.

Contraindications

It has been stated that trypsin therapy is contraindicated in patients who have abnormalities of the blood-clotting mechanism or disturbances of hepatic or renal function.

Conclusions

Intramuscular crystalline trypsin has been found to be an effective therapeutic agent in the reduction of edema resulting from trauma, hemorrhage, and infection. Its clinical applications are numerous.

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Discussion of the Paper

William M. Cooper (*New York, N. Y.*): I have accumulated in the past two years a series of about 120 cases, using trypsin in the treatment principally of either postphlebitic syndrome or thrombophlebitis of the deep or superficial veins– a large part of peripheral disease practice. Until just a few months ago I had never encountered a serious reaction with trypsin. The patient in whom this occurred was a 54-year-old man, referred to us in 1952. He was hypertensive and had large varicose veins in the left leg. He had been referred for vein surgery, but procrastinated for two years. Finally we performed the operation on May 20, 1954. I did a complete high ligation, the so-called flush ligation, getting eight tributaries at the fossa ovalis, did a complete stripping job, which was so successful that we have not had to give a single sclerosing injection since. On July 6, 1954, he came in with superficial thrombophlebitis on the posterior aspect of the right forearm. At that time we gave him some Combiotic and advised hot compresses. He did fairly well. I observed him for a short period of time. On July 21 he returned with an apparent acute superficial thrombophlebitis of the right leg; at this time it was obvious that he had developed a migrating type of thrombophlebitis affecting the superficial veins. We tried heparin. When he came back several months later with superficial thrombophlebitis of the other leg, I suggested hospitalization and anticoagulant therapy. He refused to go to the hospital, so we put him on repository heparin. He responded very well. A couple of months later he came back with another episode of thrombophlebitis. About this time I became interested in trypsin, so we started to use trypsin twice a week for three weeks, and discharged him on October 6, 1954. He had a recurrence of intermittent phlebitis in July of 1955. It cleared up very nicely with Parenzyme, and subsequently we discharged him. He came back again with thrombophlebitis in another area of the leg. On May 22 I gave him another injection. At that time I was carrying him on a maintenance dose of 0.5 cc. of Parenzyme every month. About two hours later he experienced a severe anaphylactic reaction. He responded very well to intravenous antihistamine and a little adrenalin, and recovered by evening. He came back to me on July 9 with another episode of thrombophlebitis involving the left leg and thigh and actually demanded an injection of trypsin. I was afraid to give it to him, but he insisted. With a little Chlor-Trimeton injection he survived this beautifully. He now returns once a month for treatment with trypsin because he says it makes him feel well.

A. Lee Lichtman: I think most of the reactions attributed to trypsin are due to other factors, for example, getting some material right in the vein or venule on injection. Sesame oil in the vein produces a severe reaction. The same sort of thing has happened to me on injection of most innocuous substances. I think it has been proved that it is not the trypsin that is responsible for the anaphylactic reaction; if it were, any further injection would produce an even greater reaction.

THE INTRAVASCULAR USE OF STREPTOKINASE

By Alan J. Johnson, Anthony P. Fletcher, W. Ross McCarty,
and William S. Tillett

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Streptokinase (SK) has been shown to be an effective fibrinolytic agent in animals when used intravenously alone[1-3] or in combination with a small amount of human plasma as activator.[2-5] Experimentally produced intravascular thrombi have been lysed following the production of a fibrinolytic state *in vivo*, and the obstructed vessels have become functionally patent.[1, 2, 4, 5]

The intravascular use of SK in man has been complicated by the fact that SK preparations that had been shown to be free of pyrogens in animals were often highly pyrogenic in man.[5-7] In order to prevent these complications, a study of purification methods was undertaken in this[8] and other laboratories, and biological assays of the preparations so obtained were performed to determine their toxicity. During the course of these assays, nearly 400 intravenous infusions were given to 160 patients. The results were analyzed in terms of the amount of streptokinase introduced, as well as the degree of fibrinolysis produced. Preliminary measurements were also made on each patient's blood to ensure the induction of a controlled but highly active fibrinolytic system, and appropriate precautions were observed to maintain this activity "safely" for as long as necessary.[6]

It was demonstrated previously that the older, less pure SK preparations (about 90 units/γ N) frequently evoked pyrexia, hypotension, and malaise. It was also shown that premedication of the patients with antipyretic and antihistaminic compounds suppressed these reactions.[6] The new, more highly purified SK (about 600 units/γ N) was much less toxic than the older preparations. Therefore, routine premedication with reaction-suppressing drugs was no longer needed. TABLE 1 shows the clinical effect of infusing the purified SK into patients who had no prior treatment with antipyretic or antihistaminic preparations. Infusions of I^{131}-labeled SK, of heat-denatured SK, and infusions of less than 15,000 units were excluded from this series.

It is immediately apparent that in the purified SK most of the clinical toxicity has been removed.

In view of the fact that nearly all the patients who had been infused with only 15,000 units of the impure starting material developed a temperature elevation of 2 to 3° F., it was not surprising that about 10 per cent of the patients receiving an average dose of 80,000 units of the purified SK also developed a temperature elevation of 2 to 3° F. It seemed particularly significant that one lot of the purified SK was superior to all the rest and elicited almost no clinical toxicity although 80 infusions of this lot were given to approximately 30 patients.

Since activation of the plasminogen system has been implicated in the genesis of the hypersensitivity reaction those patients with induced fibrinoly-

TABLE 1

CLINICAL EFFECT OF THE INTRAVENOUS ADMINISTRATION OF PURIFIED STREPTOKINASE IN MAN: 309 INFUSIONS IN 126 PATIENTS

Clinical effect	Dose 15,000 to 50,000 units	Dose 51,000 to 300,000 units
None	141	44
Temperature elevation of 1° F. or small fall of blood pressure	52	38
Temperature elevation of 2–3° F.* with or without fall in blood pressure	15	19

Five thousand units of starting material usually gave temperature elevation of 1° F.; 15,000 units, 2 to 3° F.

* Antipyretics were usually started when the patient's temperature was elevated to 103° F., or more.

sis might be expected to demonstrate symptoms characteristic of the hypersensitivity reaction. TABLE 2 shows the clinical effect of induced fibrinolysis in two groups of patients given SK intravenously. The control group showed no fibrinolytic activity, while the fibrinolytic group had high levels of free fibrinolytic enzyme in the peripheral blood. A very close similarity may be seen in the incidence of pyrogenic or hypotensive symptoms in both groups. The similarity in the incidence of toxic reactions was particularly striking since the patients with fibrinolysis generally received more SK than the controls. Thus, 55 per cent of the fibrinolytic group received 50,000 to 300,000 units of SK, while only 28 per cent of the control group received 50,000 to 300,000 units. No special symptoms were observed in the fibrinolytic group. These data were interpreted to mean that the toxic response to these purified SK preparations was not proportional to the dose of SK. Furthermore, it was evident that the toxicity was independent of the degree

TABLE 2

CLINICAL EFFECT OF INDUCED FIBRINOLYSIS IN PATIENTS GIVEN PURIFIED SK INTRAVENOUSLY

Clinical effect	Control group (No fibrinolysis)	Fibrinolysis group*
None	98 (52%)	41 (45%)
Rise of 1° F. or small fall of blood pressure	57 (31%)	34 (37%)
Rise of 2 to 3° F.† with or without fall in blood pressure	31 (17%)	16 (18%)
Total infusions	186	91

* Clotted whole blood taken at end of infusion from patients in fibrinolysis group was completely lysed, *in vitro*, within 24 hours at 37.5° C.

† Antipyretics were usually started when the patient's temperature was elevated to 103° F., or more.

of lysis produced and, conversely, that the fibrinolytic state caused no toxicity in man. This was also emphasized by the fact that over 80 per cent of the patients with fibrinolysis showed minimal or no symptoms.

The work of Kellner and Robertson[9] has shown that the infusion of large doses of a preparation of SK into rabbits may cause cardiac damage. To determine whether such an effect occurs in man, 165 electrocardiograms, employing all standard limb, unipolar extremity, and precordial leads were taken on 60 patients before, during, and after the infusion of 30,000 or more units of SK. Forty-seven of these patients were free from heart disease, while 13 had arteriosclerotic, hypertensive, or hypertensive-arteriosclerotic heart disease. The detailed findings cannot be considered here. TABLE 3 shows that no definite or consistent electrocardiographic changes followed the administration of streptokinase, whether or not the patient had previous evidence of severe heart disease or showed demonstrable activation of the plasminogen system. No clinical evidence of cardiac damage was noted, nor did estimation of the serum oxaloacetic transaminase levels indicate the occurrence of cardiac damage.[7]

Human sera contain variable amounts of anti-SK antibody and inhibitor(s) to the SK-plasmin system. In order to quantitate the total amount of inhibition due to their combined effect and thereby predict the amount of SK necessary to produce fibrinolysis *in vivo*, a test system was devised to measure the minimal amount of SK required to lyse the patient's clotted whole blood *in vitro* in 2 hours. This value, when multiplied by the patient's blood volume, should give the amount of SK required to produce fibrinolysis *in vivo*. A simple titration was set up, wherein freshly drawn blood was mixed with serial logarithmic dilutions of SK. The mixture was allowed to clot, as for a Lee-White clotting time, and the time of lysis noted. As shown in FIGURE 1, the reciprocals of the lysis times plotted against the log of the concentration of SK yielded a straight line, provided that only lysis times greater than 20 minutes were used. The intercept of this line at the 2-hour abscissal projection gives the concentration of streptokinase per ml. of blood required to produce clot lysis in 2 hours. This figure, when multiplied by the calculated blood volume, gives the required clinical dose.

Although this test is largely empirical, its use as a basis for prediction of dosage in 137 infusions has shown a remarkable correlation with the presence or absence of fibrinolysis at many and varied dose levels.

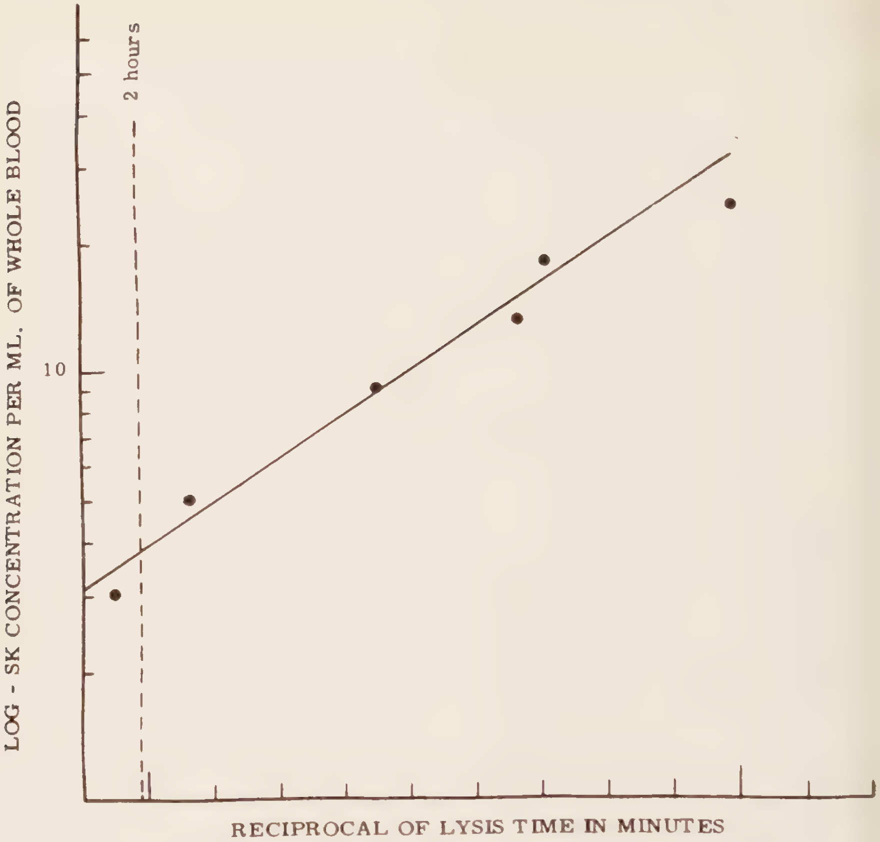
This dose-prediction test was performed on 149 patients in the hospital.

TABLE 3

ELECTROCARDIOGRAPHIC STUDIES ON PATIENTS GIVEN INTRAVENOUS INFUSIONS OF PURIFIED SK

One Hundred and Sixty Five Tracings in 60 Patients Receiving 30,000 to 200,000 Units of Streptokinase

47 patients (no cardiac disease, 114 tracings)—no change
9 patients (with heart disease, 31 tracings)—no change
4 patients (with heart disease, 15 tracings)—minor, inconsistent changes



SK CONCENTRATION AT 2-HOUR INTERCEPT x CALCULATED BLOOD VOLUME = SK DOSE.

FIGURE 1. Semilog plot of dose-prediction test on plasma from a patient prior to the intravenous infusion of streptokinase.

Statistically, these patients were a selected group, since all were hospitalized and most of them were elderly males. Probit analysis of the figures shows that the values of this test are distributed in an almost perfect log-normal array. This analysis suggests that considerable confidence can be placed in the representative nature of the data. TABLE 4 shows the distribution of dose-prediction test results in terms of clinical dosage; in these cases a blood volume of 5 liters has been assumed. For instance, a dose of 50,000 units would produce free fibrinolytic enzyme in 22 per cent of the population; 100,000 units had a similar effect in 46 per cent of the population; 500,000 units (so far, the greatest quantity administered at any one time) in 82 per cent of the population, and one million units in 92 per cent of the population. These figures are encouraging since they indicate that, initially at least, active

TABLE 4

FREQUENCY DISTRIBUTION OF DOSE PREDICTION TEST RESULTS ON 149 HOSPITAL PATIENTS

Group dose of streptokinase required for activation	No. in group	Cumulative percentage of groups
0 to 50,000 units	32	22%
50,000 to 100,000 units	37	46%
100,000 to 200,000 units	21	60%
200,000 to 500,000 units	32	82%
500,000 to 1,000,000 units	14	92%
>1 million units	13	100%

fibrinolysis can be produced in a great majority of the population by the intravenous infusion of streptokinase. However, the injection of this potent antigen may produce a late rise of antibody that would increase the dose requirement of streptokinase to such values as to render long continued infusions impracticable.

As might be expected, further quantitative studies have shown that the total inhibition, as manifest by the dose-prediction test and correlated with *in vivo* lysis, was divided into anti-SK antibody and SK-plasmin inhibitor(s) in various proportions. In an analysis of 45 samples from 37 people, balance studies have shown that the inhibition due to antibody in these samples constituted about 50 per cent of the total inhibition, with a range of 18 to 74 per cent. Expressed in units of SK per ml. of plasma, the total inhibition in different patients varied widely, with a range of 4 to 130 units, while the range of inhibition due to antibody was 1.5 to 80 units per ml. of plasma. Further studies delineating the role of each of these moieties in the activation of SK-plasmin are in progress.

Finally, it should be emphasized that all the available tools and safeguards must be repeatedly utilized toward the optimal control of fibrinolysis in the patient. Only by these means can we hope to control a strongly active, sustained, fibrinolytic system safely, for as long a period of time as it is needed

Summary

(1) The new preparations of purified streptokinase were found to be much less toxic than the old, and could be administered to patients without the reaction-suppressing drugs formerly required. Pyrogenic reactions were much less severe and hypotension became a rarity.

(2) The patients whose blood showed fibrinolysis after the intravenous infusions had no more symptoms than those patients who did not have active fibrinolysis. These symptoms did not seem to be proportional to the amount of streptokinase administered.

(3) Contrary to previous reports from investigations in animals, there was no evidence of cardiotoxicity in man, in the dosage used.

(4) A method was found to measure the total inhibition of patient sera antibody and inhibitor(s) *in vitro*. This test was used to calculate the

amount of SK needed to produce an active fibrinolytic system *in vivo* and to estimate the population susceptible to fibrinolysis with various amounts of SK. Some aspects of SK antibody and plasmin inhibitor(s), respectively, were also investigated in connection with the test.

(5) It was found that active fibrinolysis could be sustained "safely" *in vivo* for prolonged periods of time.

Acknowledgment

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Discussion of the Paper

D. L. Kline (*Yale University School of Medicine, New Haven, Conn.*): Do you have any information on the effect of SK administration upon clotting factors?

Alan Johnson: We have utilized the one-stage prothrombin test. We had at one time used the two-stage prothrombin test. W. H. Seegers and J. F. Johnson, in conjunction with us, had found that Ac-globulin and prothrombin were decreased in patients who had marked fibrinolysis. The Ac-globulin was more sensitive to fibrinolysis than the prothrombin and was usually reduced more than the prothrombin, although the latter was surprisingly sensitive to fibrinolysis. The one-stage prothrombin time was found to be very useful as an over-all guide to marked changes in these coagulation moieties when they occurred. There was no change in coagulation time nor was there evidence of thrombotic phenomenon or of bleeding at any time. We have been able to push the one-stage prothrombin time as far as 35 seconds, markedly reducing both Ac-globulin and prothrombin, without interfering seriously with coagulation. We do feel, however, that in practice it is unwise to move either of these very far. For this reason, we use the one-stage prothrombin time as a safeguard, usually allowing it to increase 3 or 4 seconds. An increase in the prothrombin time will not occur unless there is a strongly fibrinolytic system. Therefore, whole blood taken from the patient at this time and allowed to clot (as for a Lee-White coagulation time) will invariably lyse completely in less than 24 hours. Usually the clot is lysed within 2 or 3 hours.

QUESTION: I should like to ask about the inhibitor test you describe. E. E. Cliffton has written extensively on the trypsin inhibitor. It has been reported frequently that it fluctuates widely from day to day, depending upon what has been done to the patient. (Incidentally, the pregnant woman has one of the highest levels seen in a human without disease.) Do you find that you are able to demonstrate any relationship between the inhibitor level that you describe and the sedimentation rate, or other factors that have been found to be closely associated with the inhibitors of trypsin found in plasma? To give an estimate of how much of the enzyme would be required by a patient necessitates an estimate of the inhibitor level, which in turn reflects the patient's disease or his physiological status. So far as I know, the trypsin inhibitor is quite stable in normal individuals. In a large series of blood-donor plasma studies, there was found to be no difference with age. It only reflects disease.

ALAN JOHNSON: Very early, we found that the measurement of trypsin inhibitor(s) did not correlate well with our attempts to produce *in vivo* lysis in rabbits or patients. Next, we tried to determine the plasmin inhibitor, using chloroform-activated bovine plasmin as our source of enzyme. This test seemed to be equally unsatisfactory. The test that seemed to be most useful in our hands has been the one we have just described, wherein the total amount of inhibition to SK-plasmin is measured, as in the dose-prediction test. Results from preliminary studies using this same principle on plasma fractionated into inhibitor and antibody, respectively, have shown considerable promise. No attempt has been made to correlate plasmin inhibitor with the sedimentation rate, and any correlation with disease has been poor, at best. There has been some correlation with age, however, and broadly speaking, the young and the old in a general hospital population seem to have very little inhibition, while the middle-aged patients seem to have considerably more.

It is apparent that we have a great deal to learn about inhibitor and the methods for its measurement.

J. SOKAL (*Roswell Park Memorial Institute, Buffalo, N. Y.*): How long have you maintained streptokinase treatment in any single patient? What is the longest period between the first infusion of streptokinase and the last infusion the patient received?

ALAN JOHNSON: Intermittent treatment with streptokinase has been employed from 1 to 3 months or more. Therapy has been given as often as 4 to 5 times a week during this period. The patient is often allowed to rest a month or so between courses of treatment.

As far as the daily infusions are concerned, we have maintained a fibrinolytic system *in vivo* for 5 or 6 consecutive days. We usually stop Saturday or Sunday because we find the hospital does not operate quite as efficiently on Sunday: we like to watch such factors as blood pressure very carefully. There were no anaphylactoid reactions. We have found, as others have, that streptokinase is an antigenic substance, but we have not determined the precise proportion of individuals that react promptly to it as an antigenic stimulus. It is our impression that about 50 per cent do respond and that antibody formation to intravenously administered streptokinase starts on about the sixth or seventh day, as with most antibodies.

J. L. AMBRUS (*Roswell Park Memorial Institute, Buffalo, N. Y.*): Have you any information on changes in the BMR?

ALAN JOHNSON: We did not measure the BMR. It would be very interesting to see whether the BMR did go up without having elevation in temperature.

THE USE OF PLASMIN IN HUMANS*

By E. E. Cliffton

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The availability of purified proteolytic and fibrinolytic enzymes and activators has stimulated interest in their clinical use in the enzymatic debridement of wounds and abscess cavities, in the treatment of hematomas, and in the liquefaction of tracheobronchial secretions. Streptokinase-streptodornase, trypsin, pancreatic dornase, and human plasmin have been reported of value in such conditions. Streptokinase-streptodornase, trypsin, and chymotrypsin have been administered to humans intravenously.

In experimental animals streptokinase-streptodornase, chymotrypsin, and plasmin have been used to lyse thrombi both in arteries and in veins in an attempt to evaluate means of attacking directly the problem of intravascular thrombosis.

Human plasmin, a combination of activator and enzyme, has been found to be nontoxic and nonlethal in rabbits, dogs, cats, rats, monkeys, and pigs, and to produce a circulating fibrinolytic titer in all except the pig. Human plasmin has been used effectively and without serious side effects to lyse experimentally induced thrombi in the veins and arteries of rabbits, dogs, cats, and monkeys. The present report deals with data collected on the first patients given human plasmin intravenously, together with an evaluation of the various changes in clotting factors that have been observed.

Materials and Methods

Plasminogen. The plasminogen was prepared from Fraction III or III-3 of human plasma. The material used in the first patients was prepared in our own laboratory. Since then material produced by two drug companies has been used†‡.

The plasminogen was activated using a solution of streptokinase-streptodornase (SK-SD) containing approximately 10,000 units streptokinase as SK-SD per ml.§ Varying amounts of streptokinase were employed. Originally an arbitrary ratio of 1 ml. SK-SD to 10 ml. of plasminogen was used; this ratio was based on the optimum activation *in vitro* of the plasminogen as tested on the standard bovine fibrin clot. At first over 1000 units of

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‡ Supplied by Merck Sharp & Dohme, Philadelphia, Pa., through the courtesy of W. Baumgarten and R. T. Smith.

§ Varidase was supplied by Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

SK/mg. plasminogen were used, but more recently, using the new, more soluble plasminogen, 450 to 500 units SK/mg. plasminogen have been found to be sufficient.

The original plasminogen was difficult to dissolve, but recent preparations are easily soluble in small quantities of saline, distilled H_2O, or 5 per cent glucose in H_2O.

Plasmin. We used a preparation in which the plasminogen was activated in the crude state before isolation and lyophilization*.

Clinical trials. The materials were placed in solution immediately before use; the plasmin was used as dissolved, and the necessary amount of Varidase was added to the plasminogen. Using an infusion set, the plasmin in dilutions of about 0.5 mg./ml. saline was infused into the general circulation, usually via an arm vein by a slow drip. In only a very few cases was the plasmin injected directly into the affected vessels or into the thrombus itself.

Laboratory determinations. Blood samples were drawn at frequent intervals and the following determinations were made:

(1) Whole-blood clotting time (Lee-White). The capillary tube method was used only occasionally, and these instances are so recorded in the case reports.

(2) Spontaneous (whole clot) fibrinolytic activity. About 4 ml. of blood was allowed to clot in a test tube and then incubated at 37° C., and the time of complete dissolution of the clot was noted.

(3) Fibrinolytic activity. The spontaneous fibrinolytic activity of the euglobulin fraction of the plasma was measured.

(4) Proteolytic activity of the euglobulin fraction.

(5) Antiplasmin was measured in a few of the more recent cases. The method is to be reported elsewhere.

(6) Fibrinogen. Cullen and Van Slyke method.

(7) Prothrombin concentration, quick prothrombin time, Factor V, Factor VII, and prothrombin consumption were measured in the more recent cases†.

The most important questions to be answered with regard to the use of plasmin seemed to be the following:

(1) What type and severity of reactions would be obtained? Would such reactions be due to the plasminogen, to the activator (SK-SD), or to the active enzyme (plasmin)? Could these reactions be controlled?

(2) Would hepatitis be a complication of the use of this material, which is prepared from human plasma?

(3) Would plasmin itself produce an active lysis in the patient's blood or plasma? How long could fibrinolytic activity be maintained? Would repeated injections result in activity? Would proteolytic activity also be increased?

(4) Would the proteolytic and fibrinolytic activity produced within the patient's blood stream affect the homeostatic mechanism seriously; specifi-

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* Prepared by Merck Sharp & Dohme, Philadelphia, Pa.

† These studies were performed in the laboratory under the direction of Marjorie Zucker.

cally, would there be destruction of fibrinogen, clotting factors, and other proteins?

(5) One might expect that if it were possible to dissolve thrombi by this treatment, bleeding or hemorrhage might occur. Would the use of plasmin in sufficient quantity to give active lysis produce such hemorrhages?

(6) Would the development of fibrinolytic activity produce adverse effects on infections or on malignant disease?

Results

I shall report here the results produced by intravascular infusion of plasmin into 40 patients (TABLE 1). All of the early patients (1953 and 1954) were afflicted with far advanced or terminal malignant disease and had volunteered for these tests. The earlier patients treated for thrombosis or embolism were likewise suffering from malignancy or other fatal diseases. Only recently have patients without serious disease other than thrombosis or embolism been treated with plasmin. These seriously ill persons were obviously not the most satisfactory individuals for long-term evaluation, or even for detailed immediate study, and the results suffer because of this poor material; however, 10 of these patients have survived without complications for over 4 months, the longest for $2\frac{1}{2}$ years. The more recent cases will be used for most of the detailed reports.

In the first patients, relatively large quantities of SK-SD were used for activation and, because of its poor solubility, large infusion volumes were necessary. Subsequent investigations of the more soluble preparations used in the recent cases have indicated that administration of approximately 450 to 500 units SK-SD/mg. plasminogen results in optimal activation of the proactivator and proenzyme present. In the early cases, in which 3000 units SK-SD/mg. plasminogen were administered, even when only small quantities of plasmin (as little as 17 mg.) were used, severe reactions such as chills, cyanosis, headache, and high fever were obtained. With smaller amounts of SK-SD (1000 units/mg.) as activator, the reactions were less severe, but unfortunately still significant. In these cases it could not be determined whether the chill itself or another reaction might be the cause of the fibrinolytic and proteolytic activity in the patient's blood stream.

The second and larger group of patients were treated with the preactivated plasmin, the first preparation of which was very satisfactory, giving minimal reactions—slight chills and fever that lasted only a very short time and ceased even before the infusion was discontinued. However, a different preparation of this type produced not only chills and fever, occasional cyanosis and more severe reactions, but also, fairly frequently, hypotension. It is of interest, however, that even the patients with marked hypotension did not act as though they were in shock: they showed no evidence of pallor or syncope, and were even somewhat flushed; they continued to walk about, and to have a good urinary output. It appears that the hypotension was due to a moderate generalized vasodilatation that was apparently not particularly harmful. It is to be noticed that, in the preparation used in these cases, the plasminogen is activated in the crude state by SK and the plasmin then separated. An

TABLE 1
TABULATION OF ALL PATIENTS STUDIED*

No.	Patient Age	Date of treatment	Treatment No.	Diagnosis	Amount plasmin Total Mg. kg	SK-SD Total Units/ mg. pl. Units kg. pt.	Time of administration	Reaction	Euglobulin lysis time	Whole clot lysis time	Proteolytic activity Tyrosine/ ml./hr.	Fibrinogen mg. % Cullen-Van Slyke	Factor V %	Results
1	A. C. 66 W.F.	5/27/53	1	Ca. left lung A.S.H.D.	35 mg. 0.74 Plasminogen	47,000 1,343 1,000	2 hr. 30 min.	Chill Cyanosis	75 min.		0.202			Cough improved next day. Death 6 5 53. Ca. lung. Pneumonitis. Cavitation.
2	A. M. 38 W.F.	6/1/53	2	Ca. breast with metastases	17 mg. 0 3 Plasminogen	50,000 3,000 1,250	1 hr. 20 min.	Chill Fever	30 min.					Ca. breast. Pulmonary metastases. Died 7/25/53.
3	M. F. 63 W.F.	10/16/53	3	Ca. breast, P.O. Hypophysectomy Thrombosis ax. artery	200 mg. 2.3 Plasminogen	200,000 3,333 1,000	4 hr.	None	Not done	15 min.				Amputation at P.M. At P.M. had no obstruction subclavian artery. Death 11/1/53.
4	R. B. 73 W.F.	10/6/53	4	Ca. breast metastatic	70 mg. 2.1 Plasminogen	220,000 2,860 5,000	4 hr.	Chill	180 min.		0.158			Metastatic Ca. breast (acute hemorrhagic infarct kidney) P.M. Death 11/8/53.
5	S. L. 75 W.F.	Oct. 1953	5	Ca. breast Fem. art. emb. P.O. 8 hours	370 mg. 6.0 Plasminogen	370,000 1,000 6,166	3 hr.	Slight chill	9 min.	52 min.				Death. Hemolysis? Acidosis severe.
6	M. B. 48 W.F.	9/23/54	6	? Thrombosis iliac veins Pelvic metastases Ca. colon P.O., post X ray	105 mg 2.3 Plasminogen	150,000 1,430 2,490	1 hr	Slight chill	Not done	Capillary tube				No apparent improvement 24 hr.

7	G. K. 74 W.F.	10/19/54	7	Ca. pancreas Thrombo-phlebitis	115 mg. 2.3 Plasminogen	30,000 600 260		Nausea Vomiting	Not done					Relief pain while plasmin running. Edema gone. Leg soft 24 hours. Died 7/7/55 Ca. pancreas.
8	R. B. 65 W.F.	2/27/55	8	Embolus axillary artery. A.S.H.D.	225 mg. 3.2 Plasminogen	125,000 500 2,080		Vomited Chill	5 min.	<15 hr.	72			Temporary improvement. Embolus removed easily 8 hr. Living 10/30/56.
9	J. B. 73 W.F.	8/9/55	9	Femoral thromboses A.S.H.D. Diabetes Ca. colon	240 mg. 4.0 Plasminogen	100,000 150 1,666	1 hr. 30 min.			8 min.		330		8/11/55 Amputation Artery & vein occluded by old thrombus. Died P.O. for Ca. colon. Thrombosis superior mesenteric artery.
10	L. M. 62 W.M.	10/3/55	10	Ca. lung with osseous metast.	550 mg. 8	Plasmin	1 hr. 30 min.	Chill	30 min.	1.5 hr.	9.1	535–308	100%	Died 1/18/56.
11	G. P. 53 W.M.	10/17/55	11	Ca. lung with metastases general incl. brain	600 mg. 10	Plasmin	4 hr. 15 min.	Slight chill 15 min., cont'd. plasmin	120 min.	20 hr.		323–258		Died 4/19/56, P.M. Ca. lung with metastases.
12	S. F. 63 W.M.	10/20/55	12	Ca. lung ?Cerebral met.	260 mg. Plasminogen	100,000 385	2 hr.	Vomited Slightly confused	12 min.	35 min.		580–288		Living 6/10/56.
13	T. D. 65 W.M.	9/1/55	13	Ca. pancreas Bundle branch block	245 mg. 4.1 Plasminogen	100,000 410 1,666		None	120 min.	None	15.1	380–26.		Discharged 9/15/55. Died April 1956.
14	D. K. 32 W.M.	11/11/55	14	Lymphatic leuk. Phlebo-thromb.	120 mg. 1.4	Plasmin	1 hr. 30 min.	Chill, back pain, nausea	0	0	4.7	300–242		
			15		320 mg. 4.0	Plasmin	4 hr.	Slight chill	0	0	4.4	310–317		Follow-up inadequate.

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* The total amount of plasmin and the amount per kg. patient weight are shown. When plasminogen was used, the total amount of SK, the units per mg. of plasminogen and the units per kg. patient weight are given. The results recorded for fibrinolytic activity, proteolytic activity, fibrinogen and Factor V are the maximum changes seen.

TABLE 1 (*Continued*)

No.	Patient Age	Date of treatment	Treatment No.	Diagnosis	Amount plasmin Total Mg./kg.	SK-SD Total Units/ mg. pl. Units/ kg. pt.	Time of administration	Reaction	Euglobulin lysis time	Whole clot lysis time	Proteolytic activity Tyrosine/ ml./hr.	Fibrinogen mg. % Cullen-Van Slyke	Factor V %	Results
15	M. T. 78 W.M.	10/7/55	16	A.S.H.D. Failure, Embolus	200 mg. 4	Plasmin	2 hr.	Shock Same as before plasmin	40 min.	1/3 hr.	11.4	790-705		Died immediately. P.M. Cause undetermined.
16	A. T. 68 W.M.	9/27/55	17	Ca. kidney	225 mg. 4.1	Plasmin	3 hr. 15 min.	None	180 min.	Not done	6.8	335-289	100	Died 11/5/55. Ca. kidney.
17	M. G. 43 W.F.	2/5/56	18	Rh. ht. dis. Subac. bact. end. Fibrill. Embolus	650 mg. 9.2 ? (if all plasmin)	Plasmin	3 hr.	None	90 min.	0	28.5	640-633		Thrombectomy 2/5/56. Amputation 3/30/56. Living Oct 1956.
18	J. M. 62 N.M.	2/17/56	19	Terminal Ca. esoph.	200 mg. 4	Plasmin		Dyspnea Cyanosis	20 min.	1 hr.	9.3	613-400	100	Died 12 hr. P.O. Cause death bronchopneumonia?
19	T. M. 54 W.M.	2/22/55	20	Ca. lung	200 mg. 4	Plasmin	2 hr.	Chill T. 103°	34 min.	2 hr.	15.7	730-581	100-68	Died carcinomatosis 4/12/56 P.M.
		2/27/55	21		200 mg. 4		2 hr.	Chill, T. 104° Hypotension	300 min.	0	8.7	492-734	100	
20	J. G. 66 N.M.	3/5/56	22	Thrombosis retinal vein	180 mg. 2.25	Plasmin	4 hr. 15 min.	Chill, N. & V. Hypotension T. 101°	35 min.	72 hr.	11.6	506-436	100-72	Living 6 mo.
		3/7/56	23		525 mg. 6.56	Plasmin	7 hr. 20 min.	Sl. chill. T. 102° Hypotension	29 min.	16 hr.	14.3	503-475	46%-60%	No progression.
21	E. Z. 33 W.F.		24	Thrombosis retinal vein	325 mg. 5.4	Plasmin	4 hr. 30 min.	Vomited, chill T. 99°	18 min.	?	18.8	348-241	<100 to >100	
			25		100 mg. 1.6	Plasmin	1 hr.	None	32 min.	? 4 to 18 hr.	15.2	470-350		No progression.
			26		110 mg. 1.8	Plasmin	1 hr. 20 min.	Nausea	120 min.	None	9.6	512-447		Living 6 mo.

22	H. W. 52 W.F.	4/1/56	27	Ca. cervix P. O. pelvic exenteration Thrombosis femoral and iliac arteries	300	Plasmin	2 hr.	None	30 min.	Not done	13.4	566-731	84	No significant improvement.
		4/5/56	28		100	Plasmin		None	120 min.		8.6	533-600	55-50	
		4/6/56	29		200	Plasmin		None	55 min.		15.7	616-467	100-64	
		4/7/56	30		100	Plasmin		None	10 min.			473	42	
23	M. H. 42 W.F.	3/15/56	31	P.O. adrenalectomy metast. Ca. breast Thrombosis vena cava iliac & femoral Pulmonary emboli	100	Plasmin	2 hr.	Chills T. 101° Nausea	45 min.	24 hr.	8.3	560		Improvement leg. No more bloody sputum Relief chest pain.
		3/16/56	32		100	Plasmin	2 hr.	None	300 min.	None				
		3/17/56	33		100			None	240 min.	None	12.5	582		
			34		100									
24	Y. T. 68 W.F.	3/13/56	35	(1) P.O. Ca. breast	90	Plasmin	55 min.	Chills, nausea and vomiting T. 101°	18 min.	14 hr.	13.2	655-515		Relief chest pain and leg pain.
				(2) Phlebothrombosis.										Recurrence phlebothrombosis 3 weeks.
				(3) Pulmonary embolus										
			36		200	Plasmin	2 hr.	Hypotension	60 min.	None	12.4	498-501	53-38	
			37		100	Plasmin	1½ hr.	Chills, nausea and vomiting T. 102°	48 min.	None	16.2	502-428	87-64	
25	G. C.	6/13/56 AM 2:30	38	Priapism	100 mg.	Plasmin		Chill					76-84	
			39		100 mg.	Plasmin		Chill	150 min.	None	22	525	55-64	Less tense, less painful.
		6/15/56	40		100 mg.	Plasmin		Chill	7+ min.	None	15.7	672	53-56	Ultimately shown to have an abscess of penis.

TABLE 1 (*Continued*)

No.	Patient Age	Date of treatment	Treatment No.	Diagnosis	Amount plasmin Total Mg. kg.	SK-SD Total Units mg. pl. Units kg. pt.	Time of administration	Reaction	Euglobulin lysis time	Whole clot lysis time	Proteolytic activity Tyrosine / ml./hr.	Fibrinogen mg. % Cullen-Van Slyke	Factor V %	Results
26	M. P.	6/1/56 5:30	41	Cerebral accident	100 mg.	Plasmin		Record inadequate	20 hr.	None	7.0	672-727		Death 6/3/56. P.M. Hemorrhagic necrosis liver. Thrombosis middle cerebral. Perforation acute ulcer stomach.
		9:00 6/2/56 4:20	42 43		100 mg. 100 mg.	Plasmin Plasmin			Not done 9 hr. 18 hr.	None	7.0	505-742		
27	F. G. 71 W.M.	5/24/56	44	P.O. total gastrectomy Thrombosis iliac & femoral	300 mg.	Plasmin		None	60 min.	<18 hr. >4 hr.	Not done	529-348		Infusion flowed much more readily. X rays showed less dilation femoral vein. Death 5/25/56.
28	D. S. 52 W.M.	7/13/56	45	Thrombophlebitis Ca. lung metast.	100 mg. 112,000 units 2 mg./kg.	Plasmin		Chills Nausea Emesis	15 min.	1 hr.			100	Immediate relief pain calf. Living Sept. 1956.
29	T. A. 67 W.F.	6/19/56	46	Thrombophlebitis Diabetes Cerebrovascular accident 3 times Rh. Ht. dis. with aur. fibrill.	250 mg. 270,000 units 3.6 mg./kg.	Plasmin	3 hr. 30 min.	None Diabetic coma Hypotension	20 min.	2½ hr.	18.5 (13.7)	118-116	78-37	Thrombophlebitis improved.
30	G. W. 49 W.F.	9/14/56	47	Thrombosis femoral vein	25 mg. 28,000 units	Plasmin 15 min.	15 min.	None	28 min.	1½ hr.	17.1 (13.9)	370-281	42-100	Improved, discomfort less. Leg soft.
		9/15/56	48		25 mg. 28,000 units	Plasmin 10 min.	10 min.	None	280 min.	None	?	260-268	92-100	
		9/17/56	49		50 mg. 56,000 units	Plasmin 1 hr.	1 hr.	Chill	90 min.	8 hr.	14.8	462-380		
31	I. F.	10/25/56	50	Gaucher's	200 mg. 4.0 mg/kg. Plasminogen	100,000 500 2,000		None	50 min.	None	14.6	616-532		Relief pain Heparin & Dicumarol next

32	E. H. 73 W.F.	8/10/56	51	Lymphosarcoma Thrombophlebitis Thigh 3 + weeks	112,000 100 1.8	Plasmin		Chill 30 min. T. 103°	18 min.	>2 min. <11 min.	52.4	352–492	100	Marked improvement thrombophlebitis. No pain next day. Normal 8/14/56. Completely relieved by 9/12/56 with no tenderness, little redness or edema.
			52		50 0.9	Plasmin	1 hr.	None	13 min.	48 hr.	53.9	589–218	45–100	
33	E. M. 59 W.F.	9/27/56	53	Metastatic Ca breast Pulmonary emboli Repeated 5 days postirradiation	150 2.1 PP13 Plasminogen	75,000 500 mg.	1½ hr.	None	26 min.	15 hr.	19.5	715–508	50–55	P.M. No emboli in lungs. Pneumonitis. Metastatic carcinoma.
		9/28/56	54		100 1.4 Plasminogen	50,000 500 mg.	1½ hr.	None	28 min.	0	18.0	396–434	23–35	
		9/29/56	55		50 0.7 PP13 Plasminogen	500 mg.	1 hr.	None	32 min.	0	19.5	622–605	135–100	
34	R. R.	10/22/56	56	Carcinoma esophagus Terminal	200 4.0 PP13 Plasminogen	100,000 500 2,000	3 hr.	None	18 min.	35 min.		425–382	66–52	No change general condition.
35	M. D. 56 W.F.	9/29/56	57	Thrombophlebitis improved P.O. Pelvic abscess secondary to resection Ca.	175 2.5 PP13 Plasminogen	87,000 500 1,240	1 hr. 20 min.	None	19 min.	0	36.6	507–161		Less pain calf and thigh, leg softer.
		9/30/56	58		100 1.4 PP13 Plasminogen	50,000 500 714	1 hr. 45 min.	None	13 min.	<19 hr.	23.1	538–331		Improved. Continued decrease size lower leg.
		10/1/56	59		250 3.6 PP13 Plasminogen	125,000 500 1,780		None	7 min.	20 min.	22.6	548–180		
		10/2/56	60		125 1.8 PP13	6,250 500 900	2 hr. 45 min.	None	13 min.	38 min.	19.0	412–365		
		10/24/56	61		300 PP13 Plasminogen	150,000 500 2,150	2 hr. 30 min.	None	318 min.	0	8.7	326–350		

TABLE 1 (*Continued*)

No.	Patient Age	Date of treatment	Treatment No.	Diagnosis	Amount plasmin Total Mg./kg.	SK-SD Total Units/ mg. pl. Units/ kg. pt.	Time of administration	Reaction	Euglobulin lysis time	Whole clot lysis time	Proteolytic activity Tyrosine/ ml./hr.	Fibrinogen mg % Cullen-Van Slyke	Factor V %	Results
36	D. K. 57 W.F.	5/2/56 6 PM	62	P.O. Thrombosis iliac & femoral right. 24 hr. old. Ca. uterus. Pelvic exenteration 24 hr.	100	Plasmin	30 min.	None	Not done	Not done	Not done	Not done		Died 5/10/56. Post-mortem bronchopneumonia. Acute pyelonephritis No thrombosis in iliacs or femoral artery Good pulsation.
		5/2/56 1 PM	63	Thrombosis iliac left 3 hr.	150	Plasmin	1 hr.	None	29 min.		11.7	328-293	100	
37	H.G.	9/22/56	64	Thyroidectomy P.O. thrombosis carotid 3½ days	200	Plasmin	30 min.		2 min.	10 min.	12.0	508		Died 9/24/56. Lysis thrombus, good pulsation. Death 48 hr. Obstruction trachea.
38	V. P. 69 W.M.	10/23/56	65	Ca. floor mouth inoperable	25 105 3.0	Plasmin Plasminogen	2 hr.	Severe chill Cyanosis T. 103°	46 min.	12 hr.	5.3	Not done	45–100	
		11/1/56	66		250 5.3 PP13 Plasminogen	125,000 500 2,270	2 hr.	Slight chill 15 min after end infusion	30 min.	12 hr.	Not done	755-535	64–60	Abscess neck healed. Edema tongue & mouth markedly decreased.
39	N. Y. H. Pt.		67	A.S.H.D. Thrombophlebitis saphenous veins	100 Directly into thrombus	Plasmin	3 hr.							Complete lysis. Inflammation increased?
40	G. D. 66 W.M.	11/6/56	68	Ca. stomach with pulmonary metastases. Terminal	250 5.3	100,000 400 2,130	2 hr. 40 min.	None	24 min.	>9 hr.	17.7	505–388	37–80	No change. High fever, 104° F.

TABLE 2
COMPILATION OF ALL REACTIONS AND DEATHS WITHIN 24 HOURS AFTER TREATMENT

	Total treatments	Reactions	Deaths 24 hours
Plasmin	42	25 (58%)	3 (7.7%)
Plasminogen SK-SD (1000 units/mg. or more)	9	7 (77%)	1 (11%)
Plasminogen SK-SD (500 units/mg. or less)	17	1 (5.9%)	None
All types	68	33 (48%)	4 (6.0%)

excess of the streptokinase over the quantity necessary for complete activation undoubtedly remains. There is sufficient excess so that the plasmin contained in one vial, approximately 25 mg., will activate three vials of plasminogen.

When it became apparent to us that these reactions were too serious to contemplate in patients who might have coronary occlusion or pulmonary emboli, we returned to an evaluation of the amount of streptokinase used to activate the plasminogen, in order to establish whether the reactions were due to the added SK-SD. With plasminogen activated by 500 units of streptokinase mg. of plasminogen immediately before injection, we have had only infrequent minimal reactions (slight chills) and these were usually delayed until after the infusion had been discontinued. There have been no hypotensive or other significant reactions.

We have not felt that the reactions were markedly affected by antihistaminics or by cortisone administered in single doses before the plasmin was given.

The four deaths that occurred within 24 hours after the use of plasmin were in poor-risk patients (TABLE 2). Two patients (cases 5 and 27) died immediately after operation, a third (case 18) was a patient with a terminal carcinoma of the esophagus, and the fourth (case 15) was a patient who had had severe attacks of syncope and shock at least three times before the plasmin was given. Both patients who died postoperatively were difficult acid-base balance problems and it is very likely that death was caused by this condition rather than by any direct effect of the plasmin itself. Neither of these patients had an observable reaction to plasmin. The other 2 patients were very seriously ill and were expected to die momentarily; it was perhaps unwise to use them in this series.

Activity of Enzyme in the Blood

Fibrinolytic activity in the blood, as measured by the euglobulin fraction, was present in all but 2 cases studied (cases 14 and 26). Both of these patients had very high antiplasmin levels, and these levels were unaffected by the plasmin administered. It is significant that one patient (case 35) who

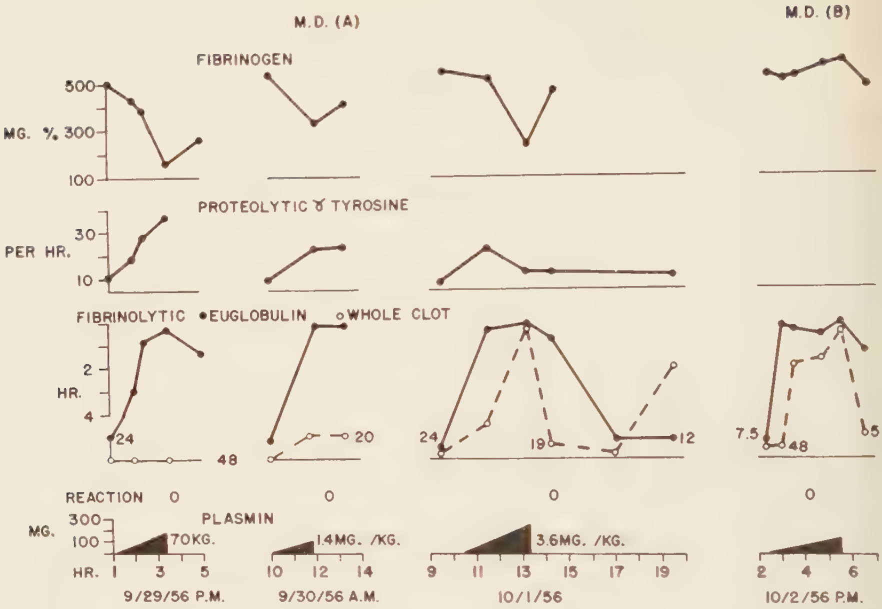

FIGURE 1. M. D., case 35. Fibrinolytic activity, proteolytic activity, and fibrinogen levels produced by 4 daily injections of plasmin. A fifth injection 22 days later was less effective because of a high antiplasmin level.

had a low antiplasmin level when given the first treatments had active euglobulin lysis times of 19, 13, 7, and 13 minutes. Twenty-two days later, when given the fifth injection, she had a high antiplasmin titer and, when given a larger amount of plasmin, had a maximum euglobulin activity of 318 minutes (FIGURE 1). The euglobulin activity returned to normal within 2 hours in all cases studied.

Whole-clot lysis was obvious in only 19 of the 52 instances in which it was determined. The longest period considered as indicating increased activity was a lysis time of 8 hours, although in most cases the control clots did not lyse in 48 hours. The maximum activity by whole-clot lysis was a lysis time of 15 minutes. Fibrinolytic activity, as measured by both methods, seemed to be increased when chills were present, but was not dependent on the presence of reactions, as shown by cases 3, 16, 17, 21, 22, 23, 27, 30, 31, 32, 33, 34, 35, 36, 37, and 40.

Proteolytic activity of the plasma, as measured by the activity of the euglobulin fraction on a casein substrate, was also increased in the majority of cases. In this test, normal human plasma usually produces less than 5.2γ of tyrosine/ml./hr. The maximum for a treated case (case 32) was 53.9γ/ml./hr.; that is, 10 times normal. It should be noted that proteolytic activity, like fibrinolytic activity, failed to rise significantly in those patients with high antiplasmin levels.

Studies were undertaken to determine whether the freeing of the proteo-

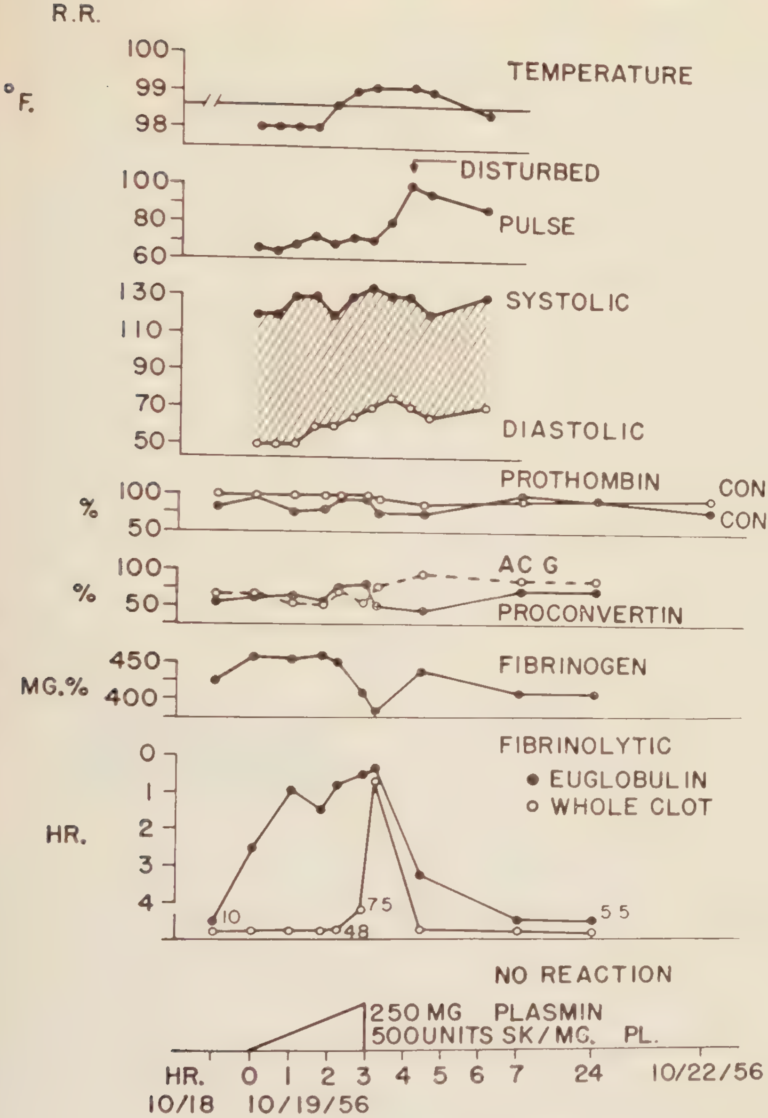

Figure 2. R. R., case 34. This chart shows the marked fibrinolytic and proteolytic activity and the moderate fall of fibrinogen produced by 4.0 mg. plasmin kg. Small changes in prothrombin time, in Factors V and VII, and in prothrombin content are shown. Blood pressure showed minimal change. There was slight temperature elevation (99° F.).

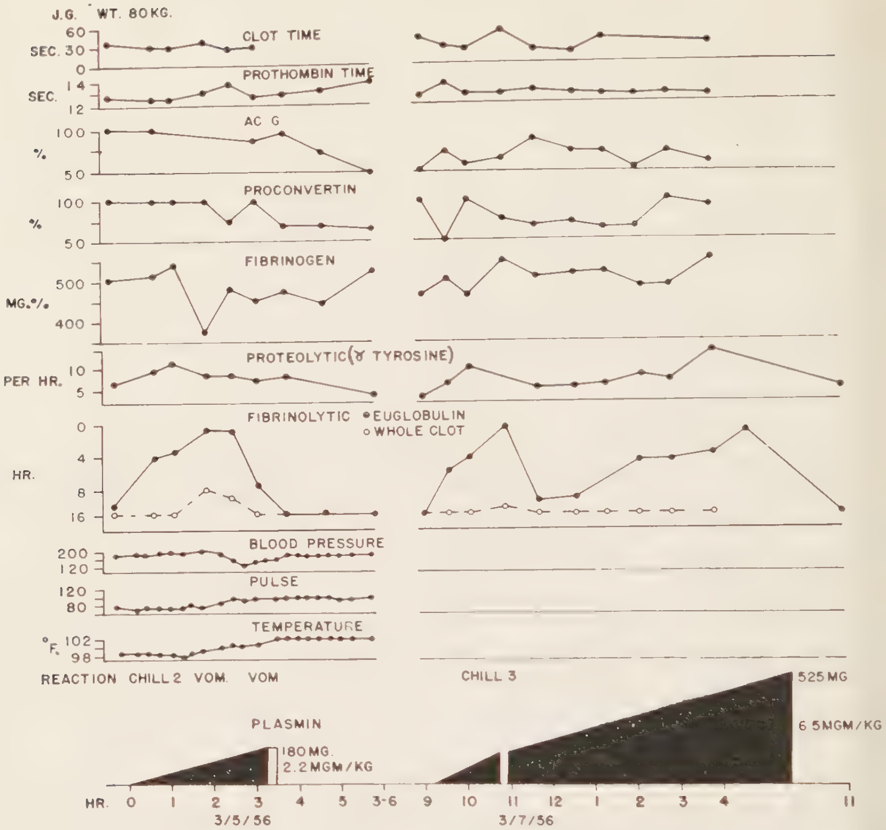

FIGURE 3. J. G., case 20. This chart depicts the same studies as FIGURE 2 in a patient with central-vein thrombosis. The data are for 2 infusions; in the second are shown the changes in the presence and absence of reaction.

TABLE 3
EFFECT OF PLASMIN ON CLOTTING FACTORS

	Fibrinogen	Prothrombin time	Factor V	Factor VII
Improved	+242 mg. %	− 1.4 sec.	+ 9%	+32%
Worse	−292 mg. %	+ 2.2 sec.	−36%	−39%
Lowest level	161 mg. %	18.4 sec.	37%	28%
No. improved	6	6	7	4
No. worse	25	9	11	4

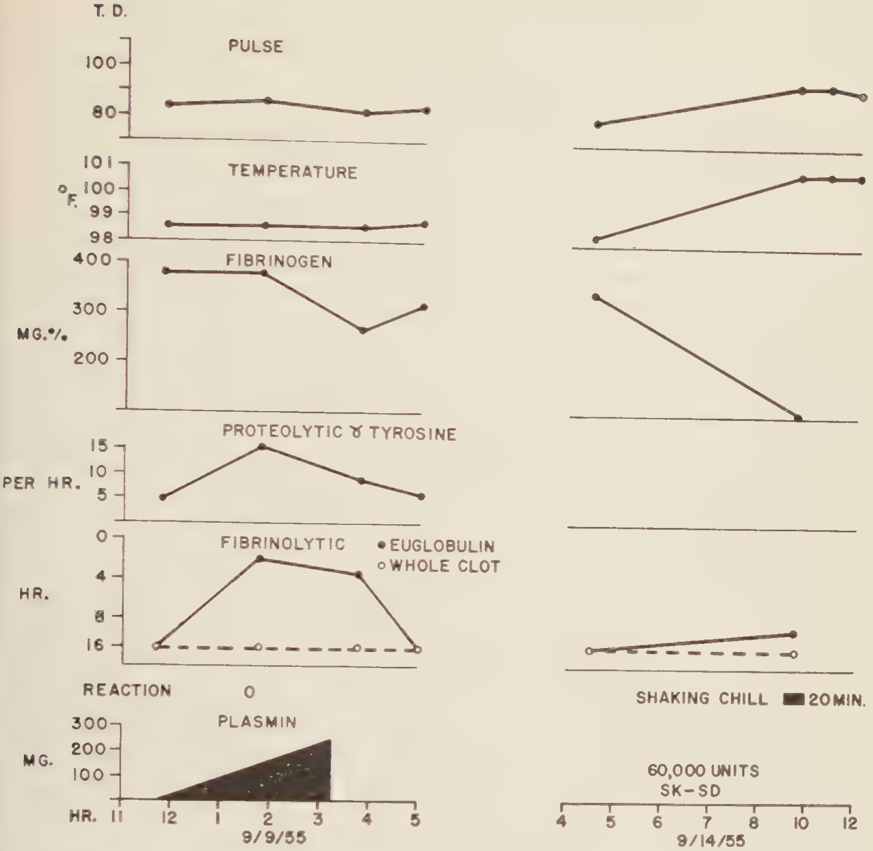

Figure 4. T. D., case 13. The effects of plasminogen activated with 100,000 units of SK as SK SD are compared with those of 60,000 units of SK as SK-SD. Note that with the SK-SD alone there is a serious reaction, with less fibrinolytic effect, and greater elevation of temperature and pulse.

lytic enzyme in the blood stream would destroy fibrinogen and other clotting factors (including prothrombin, Factor V, and Factor VII) as well as produce the desired effect of lysis of fibrin clots (TABLE 3). In the majority of cases, and especially in those with significant fibrinolytic and/or proteolytic activity, there was a drop in the fibrinogen. This was not of an alarming nature: the maximum fall was from 589 to 218, or 371 mg. per cent, and most cases fell less than 100 mg. per cent. The lowest level reached was 161 mg. per cent, a level found occasionally in otherwise normal individuals. In every instance, fibrinogen returned to normal within 12 hours after cessation of the plasmin injection. In those patients who received multiple injections of plasmin, the fibrinogen returned to normal between each treatment and, in some instances, remained above the original level during and at the end of the last treatment (FIGURE 1). It is probably significant that the greatest decreases in fibrinogen level occurred in those patients who

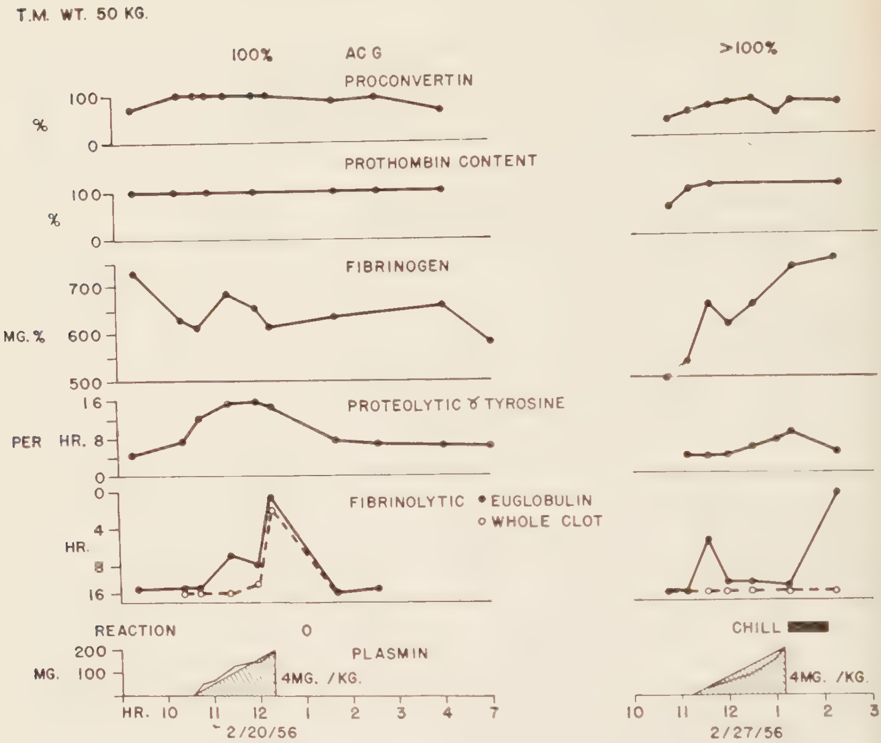

FIGURE 5. T. M., case 19. This chart indicates that in this particular patient fibrinolytic activity is as great (or greater) when there is no reaction as when a reaction occurs.

developed the highest proteolytic levels in their plasma (cases 32 and 35). Those patients who developed little proteolytic activity in general showed minimal or no decrease and, in some cases, increase in the fibrinogen level (cases 14 and 26).

There were quite marked variations in the prothrombin time, in Factor V and Factor VII, and in the prothrombin consumption tests, but these values were increased almost as often as they were decreased and in only two instances did they reach a very low level. In no case did they all fall together. The results would, however, lead one to question the advisability of combining plasmin injections with Dicumarol (3,3′-methylene bis [4-hydroxycoumarin]) or heparin; indeed, we probably have had at least one accident because of this combination (case 37). Obviously, detailed study of the combined use of these agents will be essential. Since the levels of these clotting factors return to normal within 24 hours, we have felt safe in giving Dicumerol the day after administering plasmin in a few cases, in order to prevent possible re-formation of thrombi that apparently had been dissolved. A few cases demonstrating these changes are shown in FIGURES 2 to 5.

TABLE 4

DATA ON PATIENTS TREATED FOR PERIPHERAL VENOUS THROMBOSIS

Patient Case no.	Site Type	Duration	Treatment Total (mg./kg.)		Max. lysis (min.)	Reaction	Result
			No.	Dose			
G. K. 7	Thrombosis femoral Ca. pancreas	24 hr.	1	115 (2.1) 2.1	Not tested	Nausea	Complete relief pain 6 mo. death
D. K. 14	Thrombosis? iliacs Lymphosarcoma	Days to weeks	2	120 (1.4) 320 (4.0)	0	(1) Chill, back pain (2) Slight chill	Improvement legs Improvement slight
J. M. 18	Thrombosis all forearm veins	Weeks	1	200 (4)	20	Hypotension	Complete lysis
G. C. 25	Penile vein abscess final	Weeks	3	100+ 100+ 100+	150	Chill, fever Chill, fever Chill, fever	Improvement slight
F. G. 27	Thrombosis femoral & iliac vein P.O. total gastrectomy	24 hr.	1	300 (5.0)	60	None	Easy flow after plasmin Death
D. S. 28	Thrombosis femoral	36 hr.	1	100		Chill, nausea & vomiting	Marked improvement Followed by heparin
T. A. 29	Thrombophlebitis Diabetes	?	1	250 (3.6)		Hypotension	Improved
G. W. 30	Thrombosis femoral After cutdown Weber-Osler-Rendu	48 hr.	3	25 25 50 Into femoral vein		None None Chill	Improved immediately Decreased edema Excellent 8 days Decrease in measurement 5 in.
I. F. 31	Thrombophlebitis leg	36 hr.	1	200 (4.0)	50	None	Relief pain, steady improvement Next day heparin
E. H. 32	Saphenous thrombophlebitis Leukemia	8+ weeks	2	100 (1.8) 50 (0.9)	12	Chill None	Complete cure within 48 hours
M. D. 35	Thrombosis femoral & iliac P.O. Pelvic abscess	24+ hr.	5	175 (2.5) 100 (1.4) 250 (3.6) 125 (1.8) 300 (4.2)		None None None None None	Improved Marked improvement Measurements to normal 8 days
N. Y. H. Pt. 39	Saphenous thrombophlebitis	Weeks	1	50 Direct		None	Complete lysis ? Spread infection

As in our animal experiments, the specter of hemorrhage has failed to materialize. Plasmin was given to 6 patients with recent operative wounds. Two of these (cases 22 and 36) had had a pelvic exenteration 16 and 36 hours before. One (case 27) had had a recent total gastrectomy. One (case 5) had had an attempted thrombectomy 8 hours before, and 2 others (cases 3 and 37) were not treated until more than 3 days after operation. One of these (case 3) had a pack in the pituitary fossa to control hemorrhage from the internal carotid artery. Only 2 of the 6 bled actively, one (case 22) through the punctured wound in the artery and the other (case 37) from the wound. The former had been treated with heparin also and the second with heparin and Dicumarol, obvious errors in management. One patient with Weber-Osler-Rendu syndrome (case 30), who had been bleeding actively just a few days before, failed to hemorrhage when given small amounts of plasmin. Two patients were operated upon for embolectomy within 8 hours after receiving plasmin. At operation there was no excessive bleeding, no hematoma formation in the wounds, nor any difficulty in healing. Post-mortem examinations on several patients failed to show old or new hemorrhages.

Hepatitis as a complication has not been observed. Over 50 patients, in whom plasmin was used locally in open wounds, in closed abscess cavities, and on chronic ulcers, have failed to show any evidence of hepatitis in more than 6 months of follow-up. Fifteen of the patients in this series of intravenous therapy have been followed for more than 5 months without evidence of hepatitis. Six patients in whom post-mortem examination was obtained at varying periods after treatment failed to show evidence of hepatitis.

In discussing the results on patients with thrombosis or embolism, I must stress that this is a preliminary report and that the cases are so few and

TABLE 5
DATA ON PATIENTS TREATED FOR PULMONARY EMBOLI

Patient Case no.	Site Type	No. emboli	Treatment Total (mg., kg.) No.	Treatment Total (mg., kg.) Dose	Reaction	Result
M. H.	Vena cava	4	3	100	Chills & nausea	Complete relief
23	& leg			100	None	No more emboli
				100	None	
Y. T.	Femoral	1	3	90	Chills, N. & V.	Complete relief
24				200	Chills, N. & V.	No more emboli
				100	Chills, N. & V.	Recurrence thrombosis
E. M.	Metastatic	4	4	100	None	Death
33	Ca. breast			100	None	No thrombi in pulmonary arteries
	Pulmonary			100	None	
	emboli			100	None	

have been treated with such variation in materials and techniques that no conclusions can be reached. Furthermore, anyone working in this field knows that objective proof of the site and type of thrombus or embolus is far from ideal. Particularly with pulmonary emboli and venous thromboses, there are differences of opinion concerning the results to be expected with no treatment or with conservative management.

It can be seen from TABLE 4 that venous thromboses seem to respond remarkably well to plasmin, even when there is no evidence of increased fibrinolytic activity in the blood. Relief of pain and decrease in the tenseness and in swelling are very rapid, and these improvements are followed by slow,

Table 6

Data on Patients Treated for Arterial Thrombosis or Embolism

Patient Case no.	Site Type	Duration	Treatment Total (mg./kg.)		Max. lysis	Reaction	Result
			No.	Dose			
M. F. 3	Subclavian ??	12 hr.	1	200 (2.3)		None	Temporary Amputation P.M. no thrombus Obstructed by cancer
S. L. 5	Femoral artery thrombosis P.O. Ca. breast attempted removal Heparinized	24 hr. P.O. 8 hr.	1	340 (6.0)	9 min.	None	Death No improvement leg
R. B. 8	Axillary embolus	16 hr.	1	255 (3.2)	None	Slight nausea & vomiting	Temporary Warm, motion Removal Dropped out Excellent 1½ years
J. B. 9	Femoral art. thrombosis Heparinized with cerebral reaction	At least 3 days	1	240	8 hr.	None	No improvement Amputation 2 days. Old thrombus artery and vein
M. T. 15	Popliteal thrombosis	12 hr.	1	200 (4.0)	40 min.	Shock ??	Temporary improvement Death. Embolus loose at P.M.
M. G. 17	Popliteal embolus	24 hr.	2	650 (8.0) 50 (0.9)			Temporary None. Amputation
H. W. 22	Femoral thrombosis	48 hr.	4	300 (4.9)		None	Temporary
				300 (4.9)		None	Temporary
				200 (3.3)		None	Temporary
				100 (1.7)		None	None. Amputation
D. K. 36	Iliac & femoral thrombosis Right	16 hr.	2	150 (2.5) Direct		None	Complete lysis dorsalis pedis and posterior tibial Pulses excellent
	Left			150 (2.5)		None	Improved
H. G. 37	Carotid thrombus	4 days	1	150 (2.5) Direct		None	Complete Hemorrhage, probably due to heparin & Dicumarol

but not always complete, return to normal size. In one case, with observable superficial thrombophlebitis of some weeks' duration (case 32), and in another patient (case 18), all the veins of whose forearm were thrombosed, it can be said unequivocally that the thromboses were lysed.

With pulmonary emboli, the clinical results were excellent in 2 cases (TABLE 5, cases 23 and 24). In the third, no clots were found in the pulmonary arteries at post-mortem, although the clinical diagnosis seemed definitely to be multiple pulmonary embolization. Obvious pneumonitis and metastatic carcinoma were present.

With arterial emboli and thromboses (TABLE 6) peripheral treatment with plasmin is not too promising unless much higher doses can be used. However, temporary improvement may preserve the limb (cases 3, 8, 17 and 22), and the emboli or thrombi seem to be more easily removable at operation, perhaps because of peripheral lysis (cases 8 and 15). It is conceivable that the temporary effect is due to vascular dilatation, but it may also be due to peripheral lysis of the main clot, permitting partial flow around the hard embolus, or to opening of collaterals blocked by peripheral thrombosis. Direct infusion of plasmin into the thrombosed or embolized artery via a needle or a polyethylene catheter is the method of choice in these cases. Fresh thrombi can be completely dissolved and older thrombi or emboli loosened so that they can easily be milked or aspirated from the artery at a point of choice for arteriotomy. Cases 36 and 37 are examples of this. The results observed in patients with cerebral thrombosis or thrombosis of the central vein of the eye are summarized in TABLE 7.

Advance in this field will be accelerated by the discovery of a less toxic activator, or by the development of means of removing the harmful materials from the activator itself or from the plasmin after activation. The latter approach seems the more promising.

A more thorough evaluation of the effect of inhibitors and of means of lowering the inhibitor level will be necessary. Repeated use after more than

TABLE 7

DATA ON PATIENTS TREATED FOR CENTRAL VEIN THROMBOSIS OR CEREBRAL THROMBOSIS

Patient Case no.	Site Type	Duration	Treatment Total (mg./kg.)		Max. lysis	Reaction	Result
			No.	Dose			
J. G. 20	Central vein eye thrombosis	Several days Progressing	2	180 (2.25) 525 (6.56)	29 min.	Chill, N. & V. Chill Hypotension	No progression Sight improved slightly
E. Z. 21	Central vein thrombosis	Several days Progressing past 24 hr.	3	325 (6.5) 100 (2.0) 110 (2.2)	18 min.	N. & V. Chilly None Slight nausea	No progression Sight improved slightly
M. P. 26	Cerebral	3rd episode This one several days	3	100 100 100	12 hr.	None	No change

a few days will probably result in less activity because of high inhibitor levels. How long the inhibitor remains elevated has not been determined as yet.

Summary

Studies on 40 patients who received intravenous injections of human plasmin are reported. Reactions of chills and fever were common, and hypotensive reactions were noted. Evidence has been given that these reactions were due to an excess of activator or to the particular preparation of activator used.

Significant fibrinolytic and proteolytic activity, as measured by the euglobulin fraction of plasma, was obtained in all but two of the patients adequately studied. Whole clot lysis times of significance were observed less commonly. These activities were obtained without, as well as with, chills or other reactions. Results in a few patients with venous thromboses and arterial thromboses and emboli indicated that where adequate dose levels have been reached, presumptive improvement has occurred. In three cases, definitive results have been observed.

Acknowledgment

This work would have failed completely without able and tireless help on the clinical side from Carlo Grossi, and in the laboratory from Malcolm Siegel. I am indebted to E. McDevitt of Irving Wright's laboratory and to Marjorie Zucker in our own laboratories for the studies of the clotting factors.

CLINICAL USES OF ENZYMES IN NEUROSURGERY

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Purified proteolytic enzyme preparations, recently introduced into the clinical field, represent a new and important adjuvant in the treatment of a variety of conditions. Trypsin has attracted our attention for the treatment of the sequelae that frequently accompany neurosurgical procedures and head injuries. This report presents clinical results of the administration of crystalline trypsin† in a variety of neurosurgical disorders. Furthermore, in an attempt to learn something of the mechanism of action of trypsin, a number of measurements of the clotting and lysing factors of peripheral blood were made in patients receiving this enzyme. In several cases the effects of Varidase‡ (streptokinase-streptodornase) which, although not proteolytic itself, probably acts by effecting the conversion of a proenzyme to an active protease, have been studied.

Trypsin in Intracranial Edema

The occurrence of cerebral edema following intracranial procedures is a frequent and serious complication in neurological surgery. Since one of trypsin's most effective areas of activity appears to be the reduction of edema,[1] we sought to determine whether this property of trypsin extended to the control of cerebral edema.

Following frontal craniotomy for tumors, hematomas, or other intracerebral conditions, the periorbital areas, either one or both, become so edematous that the patient may be unable to elevate his eyelids for three or four days. This periorbital edema is usually most prominent on the second postoperative day, at which time it may be difficult even for the examiner to open the patient's lids. The periorbital tissue is very firm and discolored. In instances in which intramuscular trypsin was given, the patients were able to open their lids on the second day without difficulty. The periorbital regions still appeared somewhat edematous, but the tissues were soft, the discoloration less evident, and the pupils could easily be visualized. Subconjunctival edema was almost absent.

In patients with severe head injuries, periorbital edema, lasting up to one week, may prevent observation of the state of the pupils, which is an important localizing sign for a developing intracranial expanding lesion. Following the use of trypsin the edema was considerably diminished and the pupils were easily observable within twenty-four hours following the injury. Although the periorbital tissue was somewhat enlarged when compared to the normal, there were wrinkles in the skin, indicating the rapidity with which the edema was disappearing. The same was true when edema extended to the rest of the face, as in maxillofacial injuries.

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* Charles Andrews participated in this study during the tenure of a Junior Research Fellowship awarded to him by the James W. McLaughlin Foundation, University of Texas Medical Branch, Galveston, Texas.

† The trypsin preparation used was made available to us by William Swain of The National Drug Co., Philadelphia, Pa.

‡ Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

It was our feeling that, if trypsin reduced periorbital edema, it should also reduce cerebral swelling. Therefore, we routinely placed all patients with head injuries admitted to the University Hospital under the supervision of one of us (I. J. J.) on intramuscular trypsin for a period of three days. It was our impression that, on such a regime, unconsciousness was not as prolonged as usual, but this is only an opinion and cannot be supported by objective criteria. The changes in cerebral swelling that result from severe head injuries or postoperative craniotomies are difficult to evaluate. The duration of the unconscious state is one of the best indications of the degree of edema, but this in itself varies considerably from one patient to the next. A patient with only a moderately severe injury may remain unconscious for from twenty-four hours to a week or more. There is no way to estimate the length of time any given patient would have remained unconscious had he not been treated with trypsin. Reduction of intracranial pressure and lightening of state of consciousness occur with such variability that a statistical evaluation, if not impossible, will be accomplished only with great difficulty after the study of an extremely large number of patients.

Pneumoencephalography is sometimes followed by headache, nausea, and vomiting, lasting from two to five days or longer, with gradual reduction in severity. It is more pronounced when the patient assumes an upright position than when he is supine. On the basis of this orthostatic effect, it has been suggested that the headache is produced by a hypotension within the intracranial space due to the replacement of fluid by air and a concomitant dilation of the blood vessels. The complaint is rarely encountered with a small pneumoencephalogram involving the introduction of 20 to 25 ml. of air or when the air is present in the ventricular system alone. The headache occurs only when larger amounts of air find their way into the cerebral subarachnoid space. Thus, the hypotensive effect of the air may not be the only factor in the etiology of the headache; it seems possible that air over the subarachnoid space may cause irritation, with a resultant cerebral swelling. On this basis trypsin was used intramuscularly in more than 30 patients for a period of 3 days following pneumoencephalograms. In these patients 100 to 200 ml. of air was introduced into the spinal subarachnoid space. These patients were not subjected to any other procedure such as craniotomy following the pneumoencephalogram. None of these patients had any evidence of an expanding intracranial lesion. In most cases, the final diagnosis was either posttraumatic headache or psychoneurosis; it was anticipated that such patients would continue to complain of headache for a relatively long period. The results with trypsin therapy were impressive. Many of the patients were out of bed the day following the pneumoencephalogram and some left the hospital the same day or the day following. By the third day the great majority no longer complained of headache. The reaction in a similar group of patients without trypsin was dramatically different. In these, nausea, vomiting, and severe headache were prolonged, and these reactions delayed their dismissal from the hospital.

The main disagreeable feature of intramuscular trypsin was the local pain associated with and following the injection of this material. All patients receiving trypsin complained rather bitterly of pain in their buttocks. The pain was relieved by hot tub soaks. Nevertheless, one wonders whether the improvement obtained in patients with psychoneurotic or posttraumatic headache may have been due to the diversionary pain in the buttocks.

Adhesive Arachnoiditis

Intramuscular trypsin was used in two patients with adhesive arachnoiditis. The first was a 51-year-old white female, admitted in January of 1955, with symptoms referable to acute increased intracranial pressure. Following ventriculography, a posterior fossa exploration was done, revealing an arachnoidal cyst. She improved temporarily, but soon developed symptoms of spinal-cord compression. A lumbar puncture revealed a total spinal block. The spinal fluid was deeply xanthochromic and it clotted shortly after being withdrawn from the spinal canal. After six days of intramuscular trypsin, the patient's spinal fluid was a light yellow, contained only 240 mg. per cent of protein, and did not clot on standing. The final diagnosis was that of *Cryptococcus neoformans* (*Torula*). The patient's course was progressively downhill, with involvement of the entire central nervous system, and she succumbed over a year later. Nevertheless, the response of the spinal block to trypsin was striking.

The other patient with adhesive arachnoiditis was a woman in her thirties who had had a spinal block, proved at surgery almost a year prior to the administration of trypsin. She did very well after the operation but, about eight months later, when her symptoms recurred, a myelogram again showed a complete block at the same level. She was given trypsin for over a week and obtained perhaps a slight improvement in her symptoms, but there was no change in her elevated spinal fluid protein. The adhesive arachnoiditis was a long-standing condition that was treated with the enzyme preparation only when nothing but scar tissue remained. This case should be contrasted with that of the first patient, in whom the arachnoiditis was an acute process and did respond to trypsin.

Streptokinase-Streptodornase in Infected Wounds

The effectiveness of streptokinase-streptodornase enzyme mixtures in the treatment of infected wounds is well documented.[2, 3] Illustrative of this is the case that follows. A 25-year-old white male was admitted to the Neurosurgical Service at the University Hospital in May of 1955, with a compound depressed skull fracture complicated by a pneumothorax resulting from a fractured rib. Shortly after the injury extensive debridement and elevation of skull fragments were performed and the pneumothorax was successfully treated. However, since the head wound became infected with *Staphylococcus aureus*, the wound was reopened two weeks later and the pus

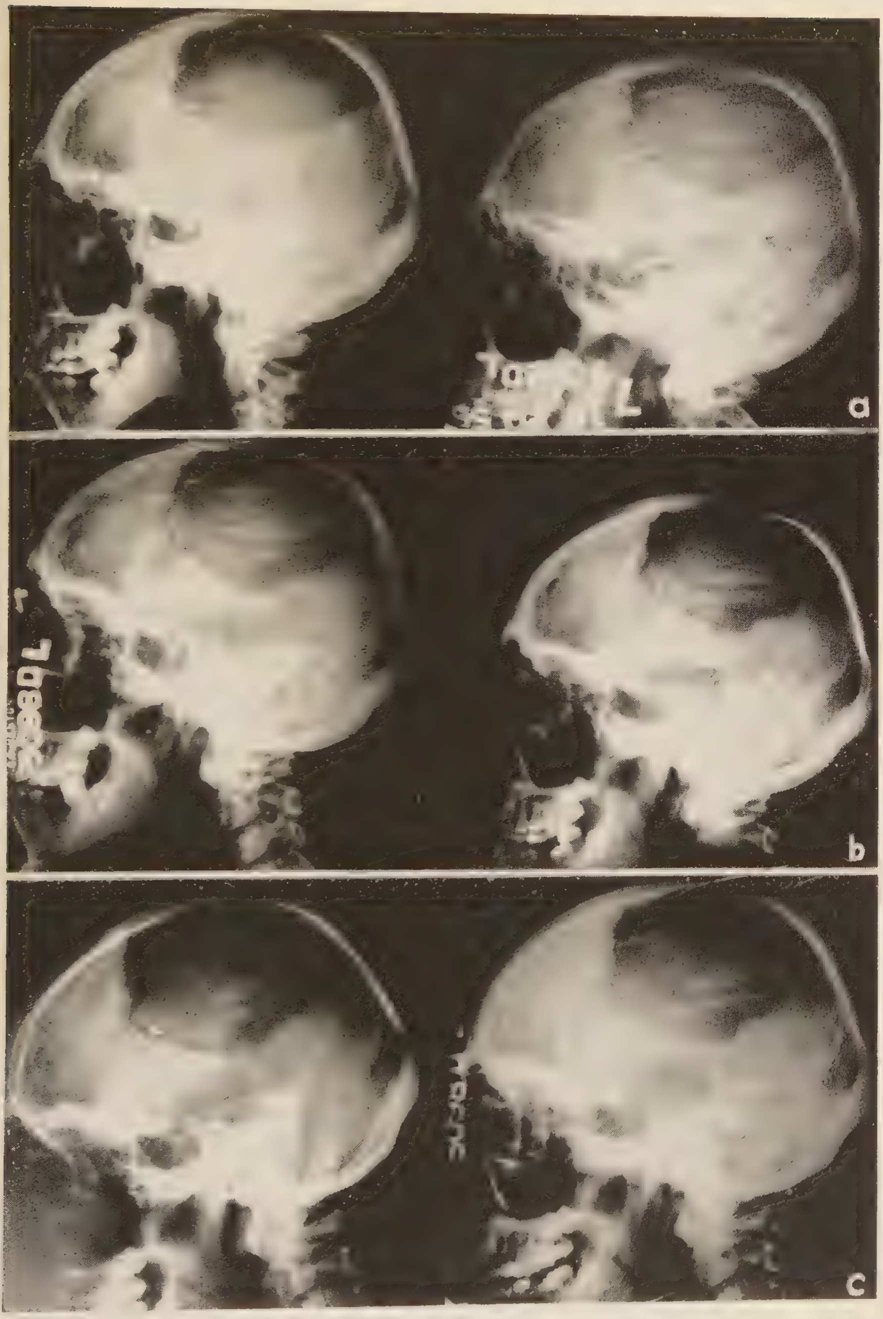

FIGURE 1. X rays of patient D. T. (a) Left, large cranial defect; right, rib graft for cranioplasty in place one month postoperatively. (b) Taken two and three months postoperatively. (c) Taken four and five months postoperatively, showing no evidence of osteomyelitis.

drained. The patient did well and was discharged three weeks after his injury. He was readmitted in November of 1955 because of progressive sensory aphasia of the right hand and some headaches. There was no evidence of infection and the previously operated site was depressed. A cranioplasty was performed in which three of the patient's own ribs were used (FIGURE 1a). Three weeks after the operation, cultures prepared from drainage of the operative wound were again positive for *Staphylococcus aureus*. Thus, we were faced with the problem of whether to remove the entire rib graft to prevent a probable osteomyelitis or to attempt local treatment of the wound. We decided to try local treatment consisting of daily topical application of the streptokinase-streptodornase enzyme mixture, Varidase, and of antibiotics for a six-week period. The wound healed, with no sequelae. According to our previous experience, such an infection would have required the total removal of the bone graft to prevent an osteomyelitis. This is especially true when the fragments of bone are loose, as they are in a graft; they become easily infected and are destroyed by the infection. However, X rays taken at monthly intervals (FIGURES 1b and 1c) indicated that these rib grafts have remained in good position without evidence of an osteomyelitis. It appears, therefore, that this graft was saved by the intervention of a proteolytic enzyme combined with antibiotics.

The next case is included to illustrate the complications that may arise from use of streptokinase-streptodornase preparations in wounds infected with β-hemolytic streptococci. The patient, a 51-year-old male, was admitted in February of 1955 with evidence of a brain tumor. Surgical excision of a cystic astrocytoma of the left frontal lobe, followed by X-ray therapy, was accomplished soon after admission. The postoperative course was uneventful and the patient continued to improve until May of 1956, when he suffered a slight contusion of the scalp in an automobile accident. A local infection resulted, with pus formation underneath the flap, necessitating incisions and debridement. β-Hemolytic streptococci were cultured from this wound. Since the literature provided with the streptokinase-streptodornase contained no specific admonition against its use in the presence of streptococcal infections, it was decided to use local applications of this preparation in the wound as in the case described above. Shortly thereafter, the patient developed a pyogenic meningitis that necessitated discontinuation of local enzyme therapy and the institution of antibiotic therapy systemically as well as locally. The meningitis quickly cleared and the wound healed satisfactorily. It seems not unlikely that the spread of the infection may have been facilitated through the action of streptokinase-streptodornase in breaking down the barrier set up in the dura against the extradural infection. This case demonstrates that proteolytic enzyme therapy must be used judiciously and that supplementary bacterial exotoxins in the presence of the bacteria that produce these exotoxins may have a harmful effect.

Peripheral-Blood Studies in Trypsin-Treated Patients

In an investigation of the effects of trypsin on blood coagulatory and lytic factors, peripheral-blood studies were performed on a number of patients receiving this enzyme. We hoped that such studies might throw some light on the chain of events that result in reduction of inflammation following intramuscular trypsin. The patients were divided into 3 groups: (1) those in whom no surgery was performed, but who received 1 ml. of trypsin intramuscularly every 6 hours for 3 days; (2) neurological patients undergoing surgery, but with no trypsin treatment; and (3) neurological patients in whom surgery was performed and who received 1 ml. of intramuscular trypsin every 6 hours for 3 consecutive postoperative days.

Venous blood samples were obtained on 2 consecutive days prior to the initiation of trypsin therapy, 6 hours after the first trypsin injection, and every 24 hours thereafter through at least the second day following its discontinuation. The following analyses were performed on each blood sample: erythrocyte sedimentation rate by the Westergren method,[4] fibrinogen,[5] prokinase,[6] antifibrinolysin,[7] latent coagulable protein,[8] Lee-White whole-blood clotting times,[9] prothrombin by the original[10] and modified[11] two-stage technique, C-reactive protein,[12] and complement titers.[13]

The detailed results of these studies will be reported elsewhere, but it may be pertinent to summarize some of them here. Following surgery without postoperative trypsin therapy there were increases in the erythrocyte sedimentation rate (ESR), fibrinogen concentrations, C-reactive protein, and complement titers. The other factors were not regularly altered in either direction. In patients receiving only trypsin, or surgery followed by trypsin therapy, the same blood components that were altered in patients undergoing surgery alone were affected, although the magnitude of the change appeared to be greater in patients receiving both surgery and trypsin. However, in the blood of patients receiving trypsin alone there appeared to be a difference in the response of several of the factors analyzed in the first sample following the initiation of trypsin administration when compared with later blood samples. In the blood taken six hours after the beginning of trypsin therapy there was a tendency for the ESR, fibrinogen, and complement titers to decrease, although all of the factors increased to a level above the pretrypsin values in all subsequent samples obtained during trypsin administration. When trypsin therapy was discontinued, the concentrations returned to essentially the pretrypsin level within two to four days.

These data fail to delineate the mechanism by which trypsin acts as an antiphlogistic agent. There is no indication here of an activation of the fibrinolytic enzyme system; if there were a significant degree of activation, changes in at least antifibrinolysin and prokinase would be expected. It therefore appears that the amounts of trypsin given to patients in these studies have not produced frank fibrinolytic activity in the circulating blood

In contrast to evidence relating to the fibrinolytic enzyme system, the direction and magnitude of alteration of a number of blood components are suggestive of a stress-type reaction during intramuscular trypsin therapy. These changes include the increase in ESR, in fibrinogen, in latent coagulable protein, in C-reactive protein, and in complement titers. However, it should not be concluded on the basis of these data alone that the trypsin response is dependent upon a stresslike reaction. The changes that occur in these blood components may be entirely independent of the sequelae that lead to the reduction in edema and inflammation during trypsin therapy. Continuation and extension of these studies will, it is hoped, help to clarify the mechanisms by which trypsin responses are mediated.

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Discussion of the Paper

Ian Thompson (*University of Texas Medical School, Galveston, Texas*): As a corollary to Dr. Jackson's paper, I should like to present some of the results we have obtained in the Urology Service at the University Hospital. For the past 8 months we have been using trypsin as adjunctive therapy in the problems of urethral stricture, and surgery of the epididymis, penis, and scrotum. The figures on urethral stricture were on individuals who on the average had to undergo dilatations at three-week intervals. We wanted to see if we could minimize the deleterious sequelae of dilatations by the administration of trypsin. We gave injections of trypsin (Parenzyme) twice daily for 1 week and then daily injections of trypsin for 3 months. In 22 patients carried for that period of time we could find no evidence of softening of strictures or any increase in the interval between dilatations. We have also used trypsin in 37 cases of epididymitis, both acute and what we call subacute. We are seeing many more of these subacute cases; frequently a patient who is already on broad-spectrum antibiotics will develop an epididymitis. Because of the rather startling pain-relieving effect of trypsin, we have not alternated cases. When trypsin therapy is instituted immediately subsequent to the diagnosis of the epididymitis, the medications for pain can be eliminated within approximately 24 hours. This is rather remarkable. For the field of penis and scrotal surgery we utilized the trypsin to prevent edema, or to attempt to assist in its prevention, and we use it in all types of hypospadias, epispadias repairs, scrotal types of surgery, hydroceles, orchidectomies, and orchioplasties. In these patients we have used trypsin in alternate cases. In the trypsin-treated patients, not only is the edema reduced, but the sanguineous discoloration has been markedly reduced. We shall continue to use this treatment in our cases.

KENNETH M. MOSER (*District of Columbia General Hospital, Washington, D. C.*): I think several things have now become clear. In the first place, many people, particularly those who have not had the opportunity to use the proteases extensively as anti-inflammatory agents, still question whether they are effective. I think those who have used them rather widely are convinced that they are, but we have very little good objective evidence to offer and we do not really know just why they do work. I should like to make a plea to carry on the type of study that Kryle reported here, and which we are now attempting to do, that is, control studies, and to include in these control studies only patients in whom the indications for anti-inflammatory therapy are clear. I believe it is well agreed that the main area for use of all these anti-inflammatory substances is acute inflammation. If we use the proteolytic enzymes in chronic conditions in which fibrosis has occurred, we shall never be certain that they are effective. Once we have established their efficacy, then we can renew our concern with how they work.

Several theories have been advanced to explain their action. The direct theory is that enzymes given at one site can reach and act at some other site. This is difficult for some of us to accept, because the quantities of enzyme are so small that they cannot even be measured. A theory that we are now considering, the validity of which we are trying to establish, is concerned with the possibility that these substances cause elevation of levels of antiprotease substances, which then affect the inflammatory site; we believe this is possible because we know that one of the basic factors in inflammation is the local release of proteases, which cause polypeptides to be released. According to recent studies, the polypeptides cause the inflammation. Some elevation of antiprotease levels does occur with injection of proteolytic enzymes, but apparently it is not sufficient to explain anti-inflammatory effect. Possibly we are not yet measuring the proper factors, but I hope that soon we shall be able to do so.

Lastly, as encouragement, I might say that our ignorance of the mechanism need not concern us too much; most of us have accepted cortisone as a reasonably effective anti-inflammatory agent, and most of us are still uncertain how it works.

INTRAMUSCULAR TRYPSIN IN CHRONIC CHEST DISEASES

By N. E. Silbert*

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Trypsin, a proteolytic enzyme, can liquefy heavy sputum, as seen in the laboratory when lysis of thick exudates is necessary in order to carry out tests.[1] Methods of direct application of trypsin and of other enzymes to human subjects have been discussed and reported in the literature.[2-3] Liquefaction of thickened secretions has been a consistent finding.

Clinical experience with inhalation of trypsin prompted an investigation of parenteral administration. Trypsin by aerosol was effective, but irritating; patients responded with copious easy raising of sputum, but developed raspy sore throats. In a previous paper,[4] I have reported the clinical results with trypsin in oil in a variety of patients with allergic chest disease, all of whom had long-standing bronchial conditions and allied pathology, including emphysema. These patients showed marked clinical improvement within two or three days after the commencement of treatment; the relief was temporary in some cases and prolonged in others. The schedule of dosage was roughly the same for all patients and consisted of daily injections intragluteally of 5 mg. of trypsin for one week, followed by three to five injections tapering off over the next week or two. In those patients who reverted in a few weeks to their original physical status, second and third courses were found almost as effective as the primary course. Side effects consisted of pain at the site of injection and, in some cases, a fine maculopapular rash around the pelvic girdle. The rash cleared on withdrawing trypsin. It was possible, however, to complete a course of treatment after an interruption of a few days. One patient developed a sensitivity to the trypsin in oil on the second course, as shown by exacerbation of her asthma; she was the only patient who did not complete the course of therapy.

Improvement consisted of thinning of the bronchial secretions within the first few days so that raising was facilitated; this was followed by a gradual diminution in the quantity of the expectorated material. Breathing was easier, usually by the second day, and it improved progressively in the succeeding days. Radiological examination before treatment indicated the degree of emphysema and of the exaggeration of bronchovascular markings. Follow-up examinations revealed changes in the bronchovascular markings, but none in the emphysema. Objective measurements were made in all patients in the series.

It is the purpose of this paper to present and to discuss these findings and to present additional data acquired from patients receiving trypsin in a different form. The new preparation, the use of which has not been reported previously, is an aqueous solution of trypsin in 5 per cent denatured gelatin. The enzyme appears to be more active in water solution than in oil, and the effect following a single dose seems to be more prolonged when the aqueous medium is used. The dilute denatured gelatin apparently exerts no depot action, but seemingly modifies the pain factor without inhibiting clinical efficacy.

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As in the previous study, the criteria for selection of subjects were: (1) long-standing chest pathology, as indicated by at least some degree of emphysema; (2) difficulty in raising tenacious mucus, and (3) the absence of associated pathology not pertinent to the study. The patients were all classified as chronic.

Methods and Materials

Thirty-five cases were studied; 25 were given trypsin in oil and 10 trypsin in the new aqueous gelatin menstruum. After physical examination, each patient had complete allergic diagnostic studies, complete blood counts, urine analysis, and posteroanterior and lateral X-ray films of the chest. In addition, the 10 patients on trypsin-gelatin and the 5 patients on trypsin in oil had vitalometer studies. Trypsin was now given once daily for 1 week and 3 to 5 times daily for the next 1 or 2 weeks. All injections were of 5 mg. and were given deep in the buttocks. After the course of treatment, the chest films and the vitalometer studies were repeated. The other studies were found to be unchanged in the first few patients and were not repeated after treatment.

The vitalometer studies included the calculation of predicted vital capacity, the measurement of the actual vital capacity, and the percentage of air expulsion during each second of expiration.

The trypsin-in-oil preparation contained 5 mg. of crystalline trypsin suspended in 1 ml. of sesame oil. To prepare trypsin in gelatin solution, 5 mg. of crystalline trypsin (lyophilized) was dissolved in 1 ml. of a 5 per cent denatured gelatin solution immediately before injection.

Results

In 10 patients who constituted the group most recently studied it was observed that the raising of inspissated mucus was facilitated. The results of the vitalometer studies on these patients are given in TABLE 1, and two representative case histories are included below. Physical changes, as disclosed by X rays, were observed in cases 1, 2, 3, 4, 5, 6, and 10. Vitalometer changes alone were observed in cases 7 and 8. However, there were either vitalometer or X-ray changes in 9 of the 10 cases; 5 of the 10 showed improvement in the vitalometer studies and 7 of the 10 in their X ray studies. The clinical improvement was more consistent than either the X-ray or vitalometer changes.

Similarly, the 25 cases previously studied were able to raise sputum more freely, and 22 of the 25 patients showed some degree of improvement in their X-ray findings. Thirteen cases were classified as markedly improved, and 9 were classified as slightly to moderately improved.

TABLE 1
VITALOMETER STUDIES OF PATIENTS TREATED WITH TRYPSIN IN GEL

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9	Case 10
Predicted vital capacity	3070 cc.	3115 cc.	3930 cc.	2970 cc.	2800 cc.	2600 cc.	2640 cc.	2400 cc.	3038 cc.	2430 cc.
Actual vital capacity	2322 cc.	2322 cc.	2529 cc.	2529 cc.	1016 cc.	1741 cc.	1555 cc.	1285 cc.	1368 cc.	1410 cc.
Rating (% below normal)	24%	25%	36%	15%	64%	33%	41%	46%	55%	41%
Air expulsion during expiration:										
First second	68%	36%	33%	79%	70%	45%	64%	48%	55%	68%
Second second	88%	82%	49%	100%	100%	64%	27%	61%	73%	92%
Third second	98%	98%	62%	—	—	79%	9%	73%	83%	100%
Fourth second	100%	100%	72%	—	—	88%	—	82%	89%	—
Recheck	(7 days later)	(7 days later)	(7 days later)	(21 days later)	(7 days later)	(31 days later)	(2 mo. later)	(1 mo. later)		(2 mo. later)
Predicted vital capacity	3070 cc.	3115 cc.	3930 cc.	2970 cc.	2900 cc.	2600 cc.	2640 cc.	2400 cc.	—	2470 cc.
Actual vital capacity	2529 cc.	2882 cc.	2488 cc.	2322 cc.	1099 cc.	1679 cc.	1949 cc.	1576 cc.	—	1679 cc.
Rating (% below normal)	11%	7%	37%	22%	62%	35%	26%	24%	—	32%
Air expulsion during expiration:										
First second	67%	78%	17%	89%	49%	12%	26%	63%	—	67%
Second second	84%	95%	33%	100%	100%	44%	62%	84%	—	99%
Third second	92%	100%	50%	—	—	66%	100%	95%	—	100%
Fourth second	97%	—	65%	—	—	78%	—	100%	—	—

Case 1: P. K., Female Registered Nurse, Aged 49

Chief complaint. Asthma, coughing, difficulty in breathing, wheezing, choking, and nasal stuffiness.

History. The patient stated that the above complaint commenced 13 to 15 years ago and that during this entire period she felt that she had not taken a complete and fully satisfying breath of air. She has had treatment over this period without any appreciable physical change.

X ray of chest. The rib cage showed widening of the interspaces. The lungs were extremely hyperaerated and emphysematous. The hilar regions were increased in width and density, and the bronchovascular markings in both bases were prominent. The diaphragms were low but sharply outlined, and the costophrenic angles were clear. The heart and superior mediastinal shadow were not remarkable.

Impression. Emphysema and peribronchial fibrosis.

Recheck X ray (18 days later). Re-examination and comparison with the previous examination disclosed a marked decrease in the width of the left hilum, as well as a decrease in the density and extent of the bronchovascular markings in the left base. The right base showed a very slight improvement.

Vitalometer studies.

Predicted vital capacity: 3070 cc.
Actual vital capacity: 2322 cc.
Patient rating: 24 per cent below normal
Air expulsion during expiration:
  First second: 68 per cent
  Second second: 88 per cent
  Third second: 98 per cent
  Fourth second: 100 per cent

Recheck vitalometer studies (7 days later).

Predicted vital capacity: 3070 cc.
Actual vital capacity: 2529 cc.
Patient rating: 11 per cent below normal
Air expulsion during expiration:
  First second: 67 per cent
  Second second: 84 per cent
  Third second: 92 per cent
  Fourth second: 97 per cent

Treatment. The procedure outlined under *Methods and Materials* was followed.

Clinical appraisal. The patient stated that for the first time in over ten years she has experienced the satisfaction of normal breathing.

Case 2: A. V., Female, Aged 36, Housewife

Chief complaint. The patient gave a history of wheezing, coughing, difficulty in breathing, and difficulty in raising sputum.

History. The patient has had severe bronchial asthma for nine years, and during the past 2 to 3 years has had frequent episodes of status asthmaticus.

X ray of chest. The rib cage was symmetrically developed and normal. The lungs were extremely hyperaerated and markedly emphysematous throughout. The bronchovascular markings were only moderately prominent, which is unusual in view of the amount of emphysema present. The diaphragms were sharply outlined and the costophrenic angles were clear. The heart and superior mediastinal shadow were not remarkable.

Recheck X rays (10 days later). Re-examination and comparison with previous examination disclosed a marked decrease in the density and extent of the bronchovascular markings in the right base. The emphysema remained the same. There was also a slight improvement in the extent of the densities in the left hilum.

Vitalometer studies.

Predicted vital capacity: 3115 cc.
Actual vital capacity: 2322 cc.
Patient rating: 25 per cent below normal
Air expulsion during expiration:
    First second: 36 per cent
    Second second: 82 per cent
    Third second: 98 per cent
    Fourth second: 100 per cent

Recheck vitalometer studies (7 days later).

Predicted vital capacity: 3115 cc.
Actual vital capacity: 2882 cc.
Patient rating: 7 per cent below normal
Air expulsion during expiration:
    First second: 78 per cent
    Second second: 95 per cent
    Third second: 100 per cent

Treatment. The procedure outlined under *Methods and Materials* was followed.

Clinical appraisal. After the third injection, the patient stated that she had marked improvement in breathing and was raising sputum with ease. She had resumed some household duties that she had been unable to perform for many months.

Discussion

The results have been discussed previously. The aqueous trypsin preparation appears to be more effective than trypsin in oil. The clinical changes are essentially the same; the difference is only in the rapidity of response. However, the major advantage of the gelatin preparation is its lack of side effects. There was a notable absence of the maculopapular girdle rash in the 10 patients treated with this preparation, as compared to 5 who developed the rash, among 25 patients given trypsin in sesame oil. There was also considerably less pain from injecting the gelatin-base trypsin. The vitalometer studies, which were not included in the previous paper, give an objective evaluation of the increased ease of breathing resulting from trypsin treatment. The differences in the vitalometer studies are significant in

5 of the 10 patients studied. There seems to be no correlation between the improvement shown by X-ray findings and by vitalometer studies.

Summary and Conclusions

Thirty-five patients with chronic bronchial pathology of long standing were given intramuscular injections of trypsin, 25 in oil and 10 in aqueous gelatin. Most patients showed definite clinical improvement. More than half showed a decrease in exaggerated bronchovascular markings in X-ray films taken before and after treatment. Vitalometer studies showed improvement in 5 of 10 patients.

The trypsin in aqueous gelatin menstruum, in contrast to the oil menstruum, caused no girdle rash and also produced less pain on injection.

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Discussion of the Paper

J. M. Warren (*Veterans Administration Hospital, Butler, Pa.*): In the last year I have treated about 41 emphysema and bronchial asthma cases with intramuscular trypsin. Unfortunately I have not had an opportunity to use the newer preparation. I agree with Silbert that the liquefaction of the inspissated mucus is remarkable. It becomes liquid so quickly that, as one patient put it, "It floods me." Another thing I noticed is the gradual decrease in the dyspnea. A very troublesome symptom with these patients is the paroxysmal morning cough; with administration of trypsin it is progressively reduced as the secretions gradually disappear. Since I have been using trypsin there is less need for ancillary drugs: I use aerosols sparingly, oxygen rarely, and vasodilators occasionally. In addition, the incidence of cross-infection has dwindled to almost nothing. I no longer see the upper respiratory infections that once were frequent.

O. H. Stuteville (*Northwestern University Medical School, Chicago, Ill.*): We have had the opportunity to use the newer preparation, trypsin in gel, and have also found it much superior.

Concluding Remarks

By O. H. Stuteville
Northwestern University Medical School, Chicago, Ill.

I should like to make a few general remarks. About two years ago we started using enzyme therapy in over 200 cases, but when we started analyzing these cases we cut them down to about 25 cases. These were all infections and traumatic injuries about the head and neck. We felt that when we had other therapy—aspirin, APC, etc.—we must discard the case. Now we are embarking on a new program. For my part, I am not convinced of everything that has been claimed for the proteolytic enzymes, but I feel that they are of definite value. I have seen some phenomenal results from this therapy. However, I feel that we must prove it to ourselves as well as to those who doubt it more than we do.

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